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# Nutrient Controls Over Cyanobacterial Synthesis of the Neurotoxin β-N-Methylamino-L-Alanine (BMAA) and Its Potential Accumulation in the Blue Crab (*Callinectes Sapidus*)

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# NUTRIENT CONTROLS OVER CYANOBACTERIAL SYNTHESIS OF THE NEUROTOXIN β-*N*-METHYLAMINO-L-ALANINE (BMAA) AND ITS POTENTIAL ACCUMULATION IN THE BLUE CRAB (*CALLINECTES SAPIDUS*)

by

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## ABSTRACT

NUTRIENT CONTROLS OVER CYANOBACTERIAL SYNTHESIS OF THE NEUROTOXIN β-*N*-METHYLAMINO-L-ALANINE (BMAA) AND ITS POTENTIAL ACCUMULATION IN THE BLUE CRAB (*CALLINECTES SAPIDUS*)

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Cyanobacteria are known to produce a variety of toxins that negatively impact both aquatic and terrestrial organisms. One putative neurotoxic compound is the non-protein amino acid β-*N*-methylamino-L-alanine (BMAA), which has epidemiological linkages to the development of several human neurological diseases. Three cyanobacterial species thought to produce BMAA —*Microcystis aeruginosa, Synechococcus bacillaris,* and *Nostoc* sp. —were grown in nutrient replete cultures to examine its synthesis and cellular distribution over a growth cycle. Production of BMAA was also examined in nutrient (nitrogen and phosphorus) deplete cultures of *Microcystis aeruginosa*. In addition, natural assemblages of phytoplankton dominated by cyanobacteria were collected from two Maryland Chesapeake Bay tributaries to determine whether natural cyanobacterial populations were producing BMAA. Blue crabs were also collected from the upper Maryland and lower Virginia Chesapeake Bay during the summer of 2018 to examine BMAA bioaccumulation in the stomach, hepatopancreas, and muscle tissues of these important benthic consumers. Concentrations of BMAA were determined via tandem high-performance liquid chromatography- mass spectrometry (HPLC-MS), a highly sensitive method that distinguishes between BMAA and analytically similar

compounds, like the structurally related but non-toxic diaminobutyric acid (DAB). Although detection limits were between 25-106 pg wet weight for cyanobacteria and were 25 pg wet weight for blue crab tissues, BMAA was not found in any sample included in this study. Further research using similarly sensitive analytical methods are needed to determine the triggers for and variability of cyanobacterial BMAA production, and its potential transfer through the food web.

I dedicate this thesis to my family, more specifically, to my mother Dr. Joanne Swift Hummel, my father Dr. Mark Hummel, and my two sisters, Katie and Dani. I cannot fully express my gratitude for their undying help and support throughout my time at ODU, especially when each of them had their own stresses with work, school, and life to manage. I dedicate this to my mother for her perpetual love and infinite availability to talk to me about any problem, anxiety, or stress I was facing. Not many people are blessed to have the kind of mother I have, and I will never truly be able to express my immeasurable gratitude to her. I dedicate this to my father for always being supportive, for listening intently to my worries, and for making light of any situation using the humor we both share. I am so lucky to have someone who cares enough to check on me weekly, send me funny animal videos, and show so much interest in my research. I thank my older sister Katie for advising me how to approach and subsequently navigate a graduate program. I truly appreciate the encouragement she has given me, beginning with graduate school applications and continuing through my final phases of the program. I also thank her for using her outstanding English skills to teach me so much about sentence structure and paper editing. Lastly, I thank my younger sister Dani for being one of my best buddies, for supporting me, joking with me, and spending time with me when I visited home. I truly cherish our laughs, similarities, and the friendship I share with both you and Katie.

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# CHAPTER I
# INTRODUCTION

## *Cyanobacteria in coastal systems*

Species of both freshwater and marine cyanobacteria are ubiquitous globally and their numbers appear to be rising due to a warming climate and coastal eutrophication (Brand et al., 2010; Carmichael, 2013; Paerl and Paul, 2012). Nitrogen and phosphorus are vital to cellular growth, but their over-enrichment in estuaries and coastal waters can result in algal blooms that can be lethal to aquatic organisms (Brand et al., 2010; Carmichael, 2013). High algal cell density results in a turbid environment which blocks sunlight necessary for macroalgal growth, coats sessile organisms, and clogs their gills and feeding apparatus (Carmichael, 2013). The death and sinking of algal assemblages can lead to hypoxic or anoxic zones as a result of bacterial respiration of algal biomass (Paerl and Otten, 2013).

Cyanobacterial blooms occur commonly in the Chesapeake Bay and its tributaries, and bloom frequency appears to be increasing, but variable, due to increased nutrient loading via terrestrial runoff and other inputs (Klemas, 2012). Harmful cyanobacterial blooms tend to dominate the Chesapeake Bay during summer months when water temperatures are warm (Gilbert et al. 2001). Nitrogen and phosphorus enrichment of warm coastal waters create conditions conducive to blooms, as cyanobacteria rapidly use the excessive nutrients to multiply (Klemas, 2012). However, due to the strong gradients of salinity, temperature, mixing, and nutrient concentrations in the Chesapeake Bay, phytoplankton distribution throughout the estuary varies by location (Harding Jr., 1994). Phytoplankton abundance in the Bay is influenced by light and nutrient availability, which vary across the Bay, and fresh-

water flow and high nutrient concentrations typically produce higher concentrations of algae (Harding Jr., 1994).

In addition to anoxia from decaying cells, many cyanobacteria produce toxins that can be consumed and transferred to higher trophic levels, where they can be biomagnified through the food web (Lobner et al., 2007; Banack et al., 2007). Some of these toxins can enter the bodies of humans through consumption of contaminated seafood, posing a threat to the human digestive system and liver, the nervous system, and other organs such as skin and mucous membranes (Carmichael, 1992; Carmichael, 1994; Chorus and Bartram, 1999; Sivonen, 2009). Microcystins are the most abundant toxin encountered in the Chesapeake Bay, and they are more frequently produced in freshwater regions (Preece et al., 2017). Tango and Butler (2008) documented toxic cyanobacteria blooms in the northern Chesapeake Bay tributaries, including the Bush, Sassaafras, Potomac, and Transquaking Rivers. They reported that in 2000, samples of *Microcystis* collected from the Sassafras River, a northern Chesapeake Bay tributary characterized by freshwater and oligohaline habitats, tested positive for microcystins (Tango and Butler, 2008). Microcystins have also been observed in the oligohaline southern Chesapeake Bay tributaries (Bukaveckas et al., 2018). In 2003, saxitoxins produced by the cyanobacteria *Aphanizomenon flos-aquae* were detected in the Sassafras River (Tango and Butler, 2008). Tango and Butler (2008) concluded that toxic cyanobacteria blooms present the largest plankton-related risk to human health each year in the Chesapeake Bay.

*Cyanobacterial toxins*

Both freshwater and marine cyanobacteria can produce a wide variety of toxins as secondary metabolites (Bláha et al., 2009). Cyanotoxins are most commonly produced by

cyanobacteria strains that thrive in fresh and brackish waters (Sivonen, 2009). There are three main toxin classes that are most harmful to animals and humans. They include hepatotoxins, affecting the liver, dermatoxins, affecting skin and mucous membranes, and neurotoxins which damage nerve tissues (Chorus and Bartram, 1999; Sivonen, 2009; Brand et al., 2010). Commonly produced hepatotoxins include microcystins and nodularins, which are typically produced by cyanobacteria living in brackish and fresh-water environments (Chorus and Bartram, 1999). Second are the dermatoxins including aplysiatoxins and lyngbyatoxins produced often by benthic marine cyanobacteria in coastal systems (Chorus and Bartram, 1999; Mazard et al., 2016). The third are the neurotoxins such as anatoxins and saxitoxins and these have been reported from water bodies in North America, Europe, and Australia and are produced by species of *Anabaena, Oscillatoria, Aphanizomenon, Lyngbya* and *Cylindrospermopsis* (Chorus and Bartram, 1999). Another putative cyanobacterial neurotoxin is β-*N*-methylamino-L-alanine (BMAA), a non-protein amino acid that has been linked to neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and Parkinson dementia complex (PDC) (Rodgers et al., 2018). Table 1 summarizes known cyanotoxins, toxin class, their producers, side effects, and toxin chemical structure.

**Table 1.** Toxin type, class, and effects produced by various cyanobacterial species.

| **Toxin Type** | **Toxin Class** | **Species** | **Toxin Effects** | **Chemical Structure** |
|---|---|---|---|---|
| Microcystins (Sivonen, 2009; Brand et al., 2010) | Hepatotoxin | *M. aeruginosa, Anabaena spp., Oscillatoria, Nostoc, Hapalosiphon, Anabaenopsis* (Carmichael, 1992; Carmichael, 1994; Rapala et al., 1997; Chorus and Bartram, 1999; Sivonen, 2009) | Hemorrhaging, liver failure, tumor production, circulatory shock, death (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) | Cyclic peptides (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) |
| Nodularins (Sivonen, 2009; Brand et al., 2010) | Hepatotoxin | *N. spumigena* (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) | Hemorrhaging, liver failure, tumor production, circulatory shock, death (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) | Cyclic peptides (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) |
| Chemically undefined (Sivonen, 2009; Brand et al., 2010) | Hepatotoxin | *Anabaena* (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) | Hemorrhaging, liver failure, tumor production, circulatory shock, death (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) | Cyclic peptides (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) |
| Chemically undefined (Sivonen, 2009; Brand et al., 2010) | Hepatotoxin | *Oscillatoria* (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) | Hemorrhaging, liver failure, tumor production, circulatory shock, death (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) | Cyclic peptides (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) |
| Chemically undefined (Sivonen, 2009; Brand et al., 2010) | Hepatotoxin | *Nostoc* (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) | Hemorrhaging, liver failure, tumor production, circulatory shock, death (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) | Cyclic peptides (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009) |
| Aplysiatoxins (Sivonen, 2009; Brand et al., 2010) | Dermatoxin | *Lyngbya* (Chorus and Bartram, 1999; Sivonen, 2009) | Tumor promotion (Chorus and Bartram, 1999; Sivonen, 2009) | Heterocyclic alkaloids (Chorus and Bartram, 1999; Sanseverino et al., 2017) |

Table 1 Continued.

| **Toxin Type** | **Toxin Class** | **Species** | **Toxin Effects** | **Chemical Structure** |
|---|---|---|---|---|
| Lyngbyatoxin-a (Sivonen, 2009; Brand et al., 2010) | Dermatoxin | *Lyngbya* (Chorus and Bartram, 1999; Sivonen, 2009) | Dermatitis, oral and gastrointestinal inflammation (Chorus and Bartram, 1999; Sivonen, 2009) | Heterocyclic alkaloids (Chorus and Bartram, 1999; Sanseverino et al., 2017) |
| Anatoxin-a, homoanatoxin-a (Sivonen, 2009; Brand et al., 2010) | Neurotoxin | *Anabaena*, *Oscillatoria*, and *Aphanizomenon* (Carmichael, 1992; Carmichael, 1994; Chorus and Bartram, 1999; Sivonen, 2009) | Paralysis of respiratory muscles, death (Carmichael, 1992; Carmichael, 1994; Chorus and Bartram, 1999; Sivonen, 2009) | Bicyclic alkaloids (Chorus and Bartram, 1999; Sivonen, 2009; Sanseverino et al., 2017) |
| Anatoxin-a(S) (Sivonen, 2009; Brand et al., 2010) | Neurotoxin | *Anabaena* (Carmichael, 1992; Carmichael, 1994; Chorus and Bartram, 1999; Sivonen, 2009) | Excessive salivation, paralysis of respiratory muscles, death (Carmichael, 1992; Carmichael, 1994; Chorus and Bartram, 1999; Sivonen, 2009) | Heterocyclic alkaloid with phosphate ester (Chorus and Bartram, 1999; Sivonen, 2009; Sanseverino et al., 2017) |
| Saxitoxins (Sivonen, 2009; Brand et al., 2010) | Neurotoxin | *Anabaena*, *Aphanizomenon*, *Lyngbya*, and *Cylindrospermopsis* (Carmichael, 1992; Carmichael, 1994; Chorus and Bartram, 1999; Sivonen, 2009) | Blockage of nerve cell sodium channels, paralysis, death (Carmichael, 1992; Carmichael, 1994; Chorus and Bartram, 1999; Sivonen, 2009) | Carbamate alkaloids (Chorus and Bartram, 1999; Sivonen, 2009; Sanseverino et al., 2017) |
| BMAA (Cox et al., 2005; Brand et al., 2010) | Neurotoxin | Various species (Cox et al., 2005; Lobner et al., 2007; Brand et al., 2010) | Neurodegenerative disease development (Cox et al., 2005; Lobner et al., 2007; Brand et al., 2010) | Amino acid (Cox et al., 2005; Lobner et al., 2007; Brand et al., 2010) |

The most commonly reported hepatotoxins are microcystins and nodularins (Table 1) (Sivonen, 2009). Microcystins are produced by cyanobacterial species *Microcystis aeruginosa* and species in the genera *Anabaena spp.*, *Oscillatoria*, *Nostoc, Hapalosiphon,* and *Anabaenopsis,* while nodularins are produced by *Nodularia spumigena* (Sivonen, 2009). Species in the genera *Anabaena, Oscillatoria*, and *Nostoc* also produce other hepatotoxins that remain chemically undefined, and on occasion they may produce other biotoxins (Carmichael, 1992).

Microcystins are very stable compounds that can persist in water for years (Chorus and Bartram, 1999). Water treatment to remove these toxins involves hydrolysis in the laboratory setting, strong oxidizing agents, and UV light degradation (Chorus and Bartram, 1999). The ability for microcystins to persist in the environment for long periods of time presents a major threat to the health of animals and humans that may be exposed to the toxins through drinking water or recreation (Rapala et al., 1997). These toxins are water soluble and uptake into cells occurs through membrane transporters that are also used to transport biochemicals and nutrients into cells (Chorus and Bartram, 1999). Microcystins also inhibit serine and threonine protein phosphatases, and therefore actively promote tumor production (Rapala et al., 1997).

Nodularins are most commonly produced in brackish waters by species of *Nodularia* (Carmichael et al., 1988; Chorus and Bartram, 1999). It is now known that nodularins are highly toxic when ingested or after exposure to contaminated water, and cause death to humans and animals typically by severe hemorrhaging of the liver (Moffitt and Neilan, 2004). They can also promote tumor production (Moffitt and Neilan, 2004).

Although there are approximately 90 known structural variants of hepatotoxins, they typically occur as cyclic peptides (Rapala et al., 1997; Chorus and Bartram, 1999; Schmidt et al., 2014). A comparison of the chemical structures of microcystins and nodularins is shown in

Figure 1. Hepatotoxins classically cause pooling of blood in the liver, internal hemorrhaging, tremors, fatal circulatory shock, liver failure, and death just hours after assimilation into animal tissue (Carmichael, 1992; Carmichael, 1994; Sivonen, 2009). Microcystins and nodularins both also promote tumor production (Sivonen, 2009).

The second cyanobacterial toxin group are the dermatoxins with examples of their chemical structures shown in Figure 2. The typical chemical structure of a cyanobacterial dermatoxin is a heterocyclic alkaloid (Sanseverino et al., 2017). Species that have been found to produce these skin irritant dermatoxins are in the genera *Lyngbya, Oscillatoria*, and *Schizothrix* (Chorus and Bartram, 1999; Sivonen, 2009). Dermatoxins include aplysiatoxins and debromoaplysiatoxin, which are tumor promoters, and lyngbyatoxin-a, which can cause dermatitis, gastrointestinal problems, and oral inflammation (Chorus and Bartram, 1999; Sivonen, 2009).

The dermatoxins aplysiatoxin and lyngbiatoxin-a, produced by the filamentous cyanobacterium *Lyngbya majuscule,* can produce severe dermatological ailments to humans when contact with the skin is made (Jiang et al., 2014b; Mazard et al., 2016). This species typically occurs as algal mats or as free-floating filamentous algae in coastal waters, and often produces enough of the toxin to cause cercarial dermatitis, or “swimmer’s itch,” a severe skin reaction causing blisters and inflammation to the skin following exposure (Jiang et al., 2014b; Mazard et al., 2016). These toxins can also cause serious gastrointestinal irritation when ingested, producing symptoms like food poisoning in humans and death to marine organisms (Jiang et al., 2014b). It has also been reported that the toxins produced by *Lyngbya majuscule* can promote tumor growth in marine organisms, like turtles and manatees (Jiang et al., 2014b).

Neurotoxins are less common than other toxin types produced by cyanobacteria and they include anatoxin-a/homoanatoxin-a (bicyclic alkaloids), anatoxin-a(S) (heterocyclic alkaloids with a phosphate ester), saxitoxins (carbamate alkaloids) (Figure 3), and BMAA (a non-protein amino acid) (Figure 4) (Chorus and Bartram, 1999; Sivonen, 2009; Sanseverino et al., 2017).

Originally found to be produced by *Anabaena flos-aquae*, it is now known that anatoxin-a is produced by several *Anabaena* species including *A. circinalis, A. planctonica, A. spiroides,* as well as species in the genera *Aphanizomenon, Cylindrospermum, Planktothrix,* and *M. aeruginosa* (Rastogi et al., 2015). Its homologous compound, homoanatoxin-a, is produced by *Oscillatoria formosa, Phormidium formosum,* and *Raphidiopsis mediterranea,* and species in the genera *Anabaena*. Both toxins interfere with normal nervous system functioning and can cause paralysis of respiratory muscles and subsequent death within minutes of exposure (Carmichael, 1992; Chorus and Bartram, 1999; Rastogi et al., 2015). These two alkaloid neurotoxins are known as fast death factors (FDF), meaning they are fast acting on the nervous system once an organism is exposed (Rastogi et al., 2015). These neurotoxins are particularly dangerous for animals and humans as they cannot be degraded by any enzyme found in eukaryotic cells, and the rapid muscle contraction it causes can quickly lead to paralysis and death (Carmichael, 1992). Anatoxin-a(S), a variant of anatoxin-a, is also produced by *Anabaena* and can cause similar ailments, and excessive salivation in animals (Carmichael, 1992).

Saxitoxins are unique compounds as they are produced by two completely different organisms that thrive in marine and freshwater systems (Cusick and Sayler, 2013). More commonly known as products of dinoflagellates in marine systems, saxitoxins can be

produced by cyanobacterial species in the genera *Anabaena*, *Aphanizomenon*, *Lyngbya*, and *Cylindrospermopsis*, typically in freshwater systems (Carmichael, 1992; Chorus and Bartram, 1999; Cusick and Sayler, 2013). These tricyclic carbamate alkaloids exist in 27 different forms, and are extremely potent toxins (Rastogi et al., 2015). Saxitoxins block nerve cell ion channels and cause an interference between muscle and nerve cells (Sivonen, 2009; Hackett et al., 2013). They can also accumulate in the tissues of aquatic organisms and cause paralytic shellfish poisoning (PSP) in human consumers by binding to sodium, potassium, and calcium channels and inhibiting the movement of these ions in nerve and muscle cells, causing eventual death by paralysis (Sivonen, 2009; Hackett et al., 2013). Initial symptoms of saxitoxin exposure in humans include numbness in the face and neck, muscle weakness, ataxia, confusion and trouble breathing, and in extreme cases, paralysis and death (Cusick and Sayler, 2013). Additionally, if saxitoxins are not harmful to primary consumers of the cyanobacteria, and even secondary consumers of those organisms, there is the potential for bioaccumulation in higher trophic level organisms (Cusick and Sayler, 2013).

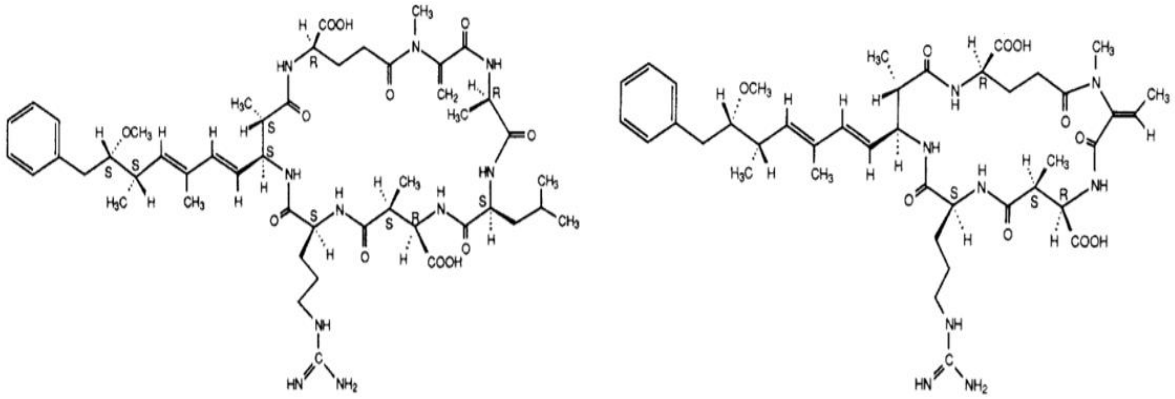

**Figure 1.** Chemical structure of microcystin (left) and nodularin (right) (image from Rinehart et al., 1988).

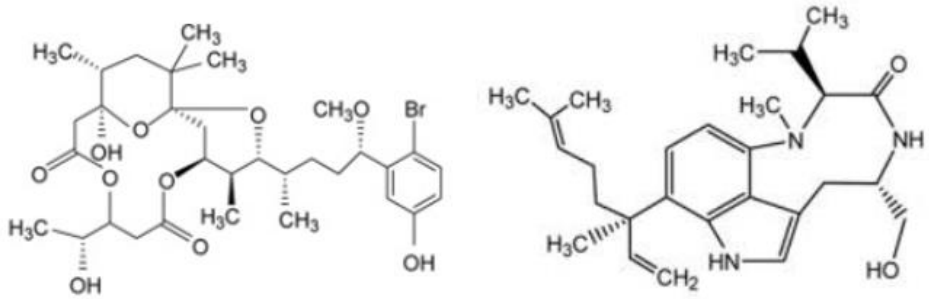

**Figure 2.** Chemical structure of aplysiatoxin (left) and lyngbyatoxin (right) (image from Rastogi et al., 2015).

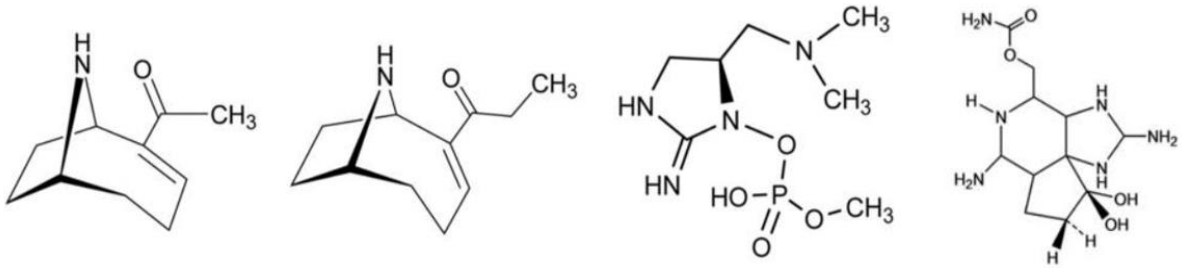

**Figure 3.** Chemical structures (from left to right) of anatoxin-a, homoanatoxin-a, anatoxin-a(S), and saxitoxin (image from Rastogi et al., 2015).

In addition to known toxins of cyanobacteria, one neurotoxin has been putatively linked to the development of several neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) and Parkinson dementia complex (PDC) (Field et al., 2013). This toxin is the non-proteinogenic amino acid BMAA (Lobner et al., 2007; Brand et al., 2010). Unlike the 20 proteinogenic amino acids, BMAA is one that is not directly involved in the formation of proteins (Fipke and Vidal, 2016). Unlike the fast-acting alkaloid cyanobacterial neurotoxins that block varying ion channels in neurons and bind to acetylcholine receptors in humans, BMAA accumulates in neuronal cells over time by binding to glutamate receptors and incorrectly incorporating into proteins (Rodgers et al., 2018). The misincorporation of BMAA into proteins causes subsequent misfolding and entanglement of neurons, producing similar symptoms as those seen in patients of ALS/PDC (Rodgers et al., 2018). This may explain why the symptoms of BMAA toxicity are often chronic, as time is required for weak proteins to aggregate in neurons and slowly inhibit proper neuron function (Rodgers et al., 2018). BMAA is also bioaccumulated in the tissues of organisms that directly or indirectly consume it, and so the potential for trophic transfer and biomagnification in higher organisms is great (Rodgers et al., 2018). This is of concern to human consumers of commercial aquatic organisms since they will have accumulated the greatest concentrations of BMAA (Rodgers et al., 2018).

*Neurotoxic pathways of BMAA*

Although previous studies suggest that nearly all cyanobacteria species produce BMAA, the reasons for its production remain unknown, though it has been speculated that its production is a function of nutrient availability and life cycle stages (Cox et al., 2005; Esterhuizen and Downing, 2008). Cox et al. (2005) reported that nearly all cyanobacterial

groups tested from a diverse array of environments (marine, brackish, freshwater, terrestrial, etc.) were capable of producing BMAA (Table 2). Cellular concentrations of BMAA ranged from 3 to 6478 μg/g free and 4 to 5415 μg/g protein-bound BMAA, and these concentrations were obtained using HPLC separation and fluorescence detection methodologies. However, this would suggest that potential human exposure would be nearly ubiquitous as well.

Although BMAA is not concentrated in lipids, it still has the potential to bio-magnify through the food web, posing a threat to consumers of contaminated commercially important marine organisms, like blue crabs (*Callinectes sapidus*) (Brand et al., 2010). There are several proposed mechanisms by which BMAA accumulates in the tissues of higher trophic level organisms, each relating to BMAA binding to proteins in these organisms after introduction into the body. Banack et al. (2007) stated that although BMAA is non-proteinogenic, it is not unusual for this amino acid to be associated with and stored in cyanobacterial proteins, allowing for biomagnification and slow release of the toxin over time. The incorporation and storage of BMAA in proteins functions as a neurotoxic reservoir, allowing for intermittent release directly into human brain tissue (Banack et al., 2007). During protein synthesis, BMAA can be incorrectly inserted into proteins as a foreign amino acid, causing protein mutations, leading to neurodegeneration of brain cells over time, and subsequent disease development (Figure 5) (Murch et al., 2004; Banack et al., 2007; Holtcamp, 2012). The hypothesis is that because BMAA mimics structurally similar essential amino acids such as glutamic acid, it can be misincorporated into proteins at glutamate receptors, causing them to misfold (Gregersen et al., 2006; Banack et al., 2007). Additionally, it has been shown that BMAA can replace serine and alanine during protein synthesis in humans (Jiang et al., 2014a).

Glutamate receptors are excitatory amino acid receptors, and when activated by BMAA, lead to excitotoxicity and subsequent cell death (Diaz-Parga et al., 2018). There are two main subdivisions of excitatory amino acid and glutamate receptors, and they include ionotropic receptors (NMDA and AMPA or iGluRs) and metabotropic glutamate receptors (mGluRs) (Diaz-Parga et al., 2018). A strong increase in glutamate or structurally similar compounds can overstimulate the receptors with $Na^{+}$ and $Ca^{2+}$, causing receptor dysfunction, and lead to neuronal apoptosis and eventual neurodegeneration (Diaz-Parga et al., 2018). Delcourt et al. (2018) proposed that BMAA binds to and activates glutamatergic receptors iGluR and mGluR, which leads to an increase in $Ca^{2+}$ (Figure 6). The additional $Ca^{2+}$ coupled with the hyperphosphorylation of the Tau protein leads to endoplasmic reticulum stress, tangle degeneration, and apoptosis (cell death) (Delcourt et al., 2018). Additionally, the overload of $Ca^{2+}$ can lead to mitochondrial dysfunction and the production of reactive oxygen species (ROS), causing cell death (Van Den Bosch et al., 2006).

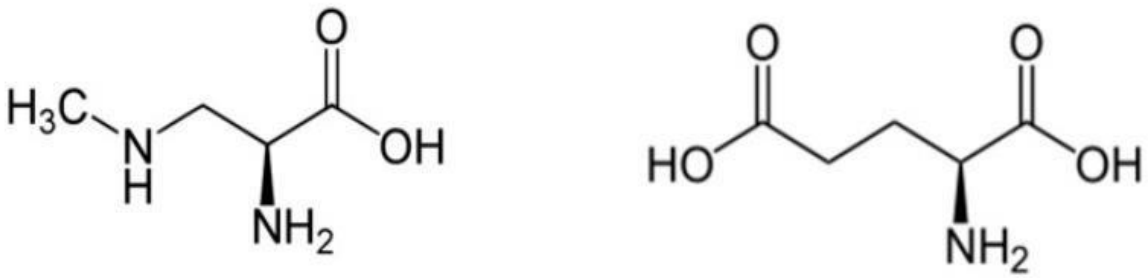


**Figure 4.** Chemical structure of BMAA (left) and glutamate (right) (image from Delcourt et al., 2018).

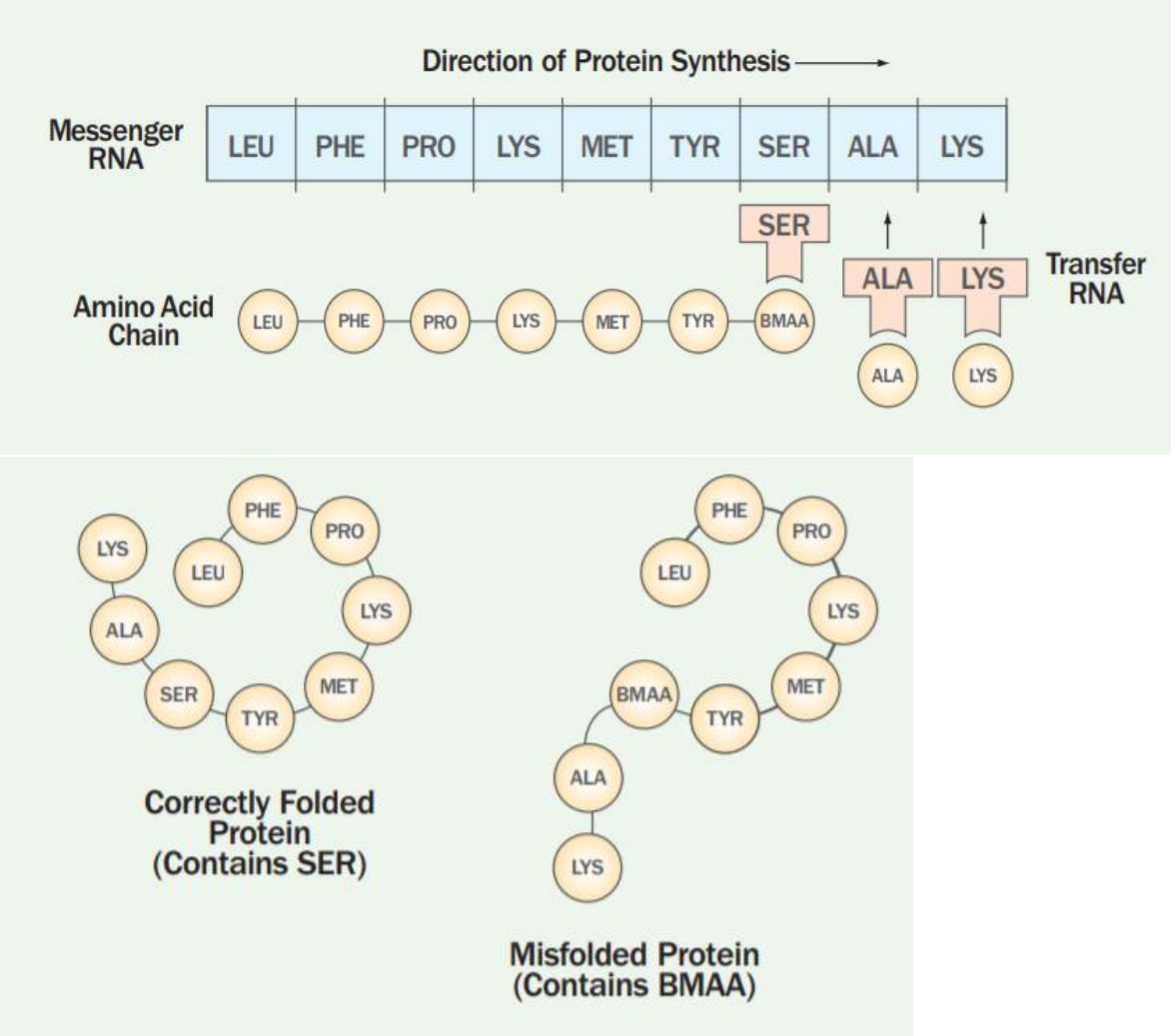


**Figure 5.** An illustration of protein synthesis (top). BMAA in the amino acid chain binds to serine in transfer RNA and becomes part of the protein chain. An illustration of a correctly folded protein containing serine and an incorrectly folded protein containing wrongly incorporated BMAA are shown (bottom) (images from Holtcamp, 2012).

**Table 2.** BMAA detected in cyanobacteria species and their corresponding habitats/origins (table is adapted from Cox et al., 2005 (and references therein), Table 2 "BMAA in free-living cyanobacteria"). All BMAA concentrations were obtained using HPLC and fluorescence detection.

| **Cyanobacterial strain** | **Habitat** | **Origin** | **Free BMAA (µg/g)** | **Protein BMAA (µg/g)** |
|---|---|---|---|---|
| *Microcystis* PCC 7806 | Freshwater | The Netherlands | 4 | 6 |
| *Microcystis* PCC 7820 | Freshwater | Scotland | 6 | 12 |
| *Prochlorococcus marinus* CCMP1377 | Marine | Sargasso Sea | 32 | 57 |
| *Synechocystis* PCC 6308 | Freshwater | U.S.A. | - | - |
| *Synechocystis* PCC 6301 | Freshwater | U.S.A. | 25 | - |
| *Chroococcidiopsis indica* GQ2-7 | Marine coral | Unknown | 436 | 76 |
| *Chroococcidiopsis indica* GT-3-26 | Marine rock | Unknown | 1306 | 5415 |
| *Myxosarcina burmensis* GB-9-4 | Marine coral | Marshall Islands | 79 | 1943 |
| *Myxosarcina concinna* GT-7-6 | Marine coral | Unknown | 1501 | 960 |
| *Lyngbya majuscula* | Marine | Zanzibar | 32 | 4 |
| *Planktothrix agardhii* NIES 595 | Freshwater | Northern Ireland | 318 | 30 |
| *Plectonema* PCC 73110 | Unknown | Unknown | 155 | 150 |
| *Phormidium* | Unknown | Unknown | 11 | 270 |
| *Symploca* PCC 8002 | Marine, intertidal | U.K. | 3 | 262 |
| *Trichodesmium thiebautii* | Marine | Caribbean | 145 | 8 |
| *Trichodesmium* CCMP1985 | Marine, coastal | North Carolina | 13 | 17 |
| *Anabaena* PCC 7120 | Unknown | U.S.A. | 32 | - |
| *Anabaena variabilis* ATCC 29413 | Freshwater | U.S.A. | 35 | - |
| *Aphanizomenon flos-aquae* | Marine | Baltic Sea | - | 866 |
| *Cylindrospermopsis raciborskii* CR3 | Freshwater | Australia | 6478 | 14 |
| *Nodularia spumigena* | Brackish water | Baltic Sea | 16 | 50 |
| *Nodularia harveyana* CCAP 14521 | Marine | Unknown | 20 | 11 |
| *Nostoc* 268 | Brackish water | Baltic Sea | 34 | 274 |
| *Nostoc* PCC 6310 | Freshwater | Israel | 42 | - |
| *Nostoc* PCC 7107 | Freshwater | U.S.A. | 27 | 1772 |
| *Nostoc* sp. CMMED 01 | Marine | Hawaiian Islands | 1243 | 1070 |
| *Calothrix* PCC 7103 | Unknown | Unknown | 13 | 92 |
| *Chlorogloeopsis* PCC 6912 | Soil | India | 758 | - |
| *Fischerella* PCC 7521 | Yellowstone, hot spring | U.S.A. | 44 | 175 |
| *Scytonema* PCC 7110 | Limestone cave | Bermuda | - | 1733 |

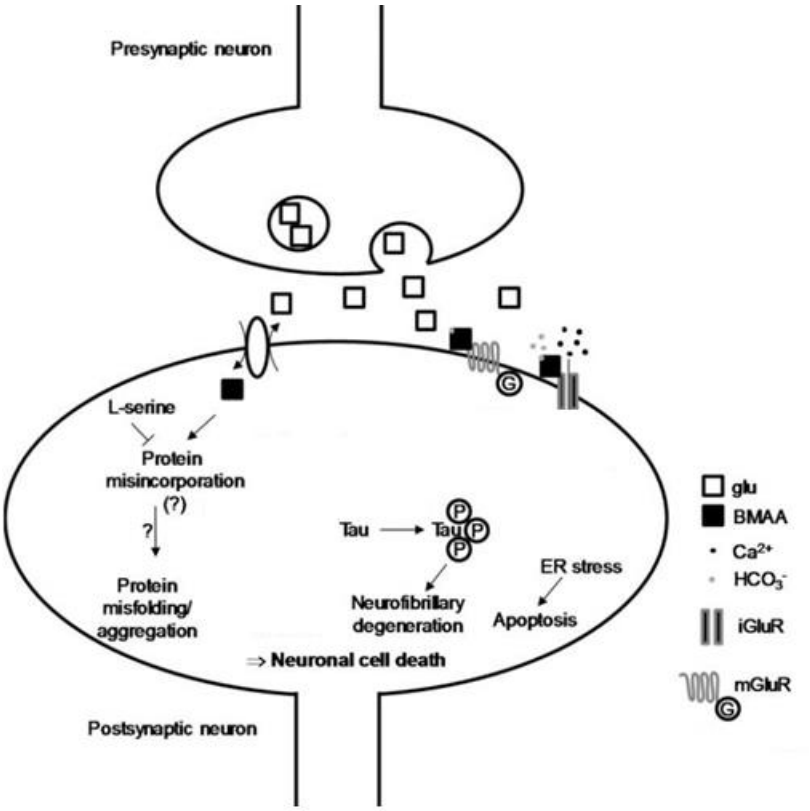


**Figure 6.** Proposed mechanism for BMAA binding to glutamate receptors at the glutamatergic synapse, leading to an increase in $Ca^{2+}$ and subsequent excitotoxicity (neuronal death) (image adapted from Delcourt et al., 2018).

*The potential for BMAA accumulation and biomagnification*

One of the first reported cases of BMAA transfer through the food web involved the Chamorro people of Guam, who's diet included flour made from cycad seeds (from *Cycas micronesica*), of which the cyanobacterium *Nostoc* is a root endosymbiont (Cox et al., 2003). However, for BMAA to accumulate in the tribe members in great enough quantities to cause neurological diseases, they would have had to consume enormous amounts of the flour (Cox et al., 2003). It was then proposed that the BMAA was biomagnifying through another consumer of the cycad seed, the flying fox, *Pteropus mariannus*, which the Chamorro tribe

also consumes (Cox et al., 2003). It was ultimately determined that *Nostoc* was the source of the BMAA production in the seeds (Cox et al., 2003).

Due to the ubiquity of cyanobacteria and their toxins in marine and aquatic systems, there is significant potential for accumulation of BMAA in the tissues of organisms that feed on them. BMAA is thought to accumulate as a protein-bound form and a freely cellular form (Murch et al., 2004). The protein-bound form of BMAA has been proposed to function as a neurotoxic reservoir that is transferred throughout the food web allowing for the slow release of BMAA over time during digestion and protein metabolism (Murch et al., 2004; Jiang et al., 2014a). Cox et al. (2003) reported biomagnification of BMAA in the Chamorro food chain, finding higher BMAA concentrations with each trophic level, from cyanobacteria (0.3 μg/g), to seed (2-37 μg/g), to flying fox (3556 μg/g). This trophic transfer of BMAA has been proposed to be the cause of the widespread development of neurological diseases, such as ALS and PDC, amongst the Chamorro tribe (Murch et al., 2004).

A study performed by Jonasson et al. (2010) showed the trophic transfer of BMAA from cyanobacteria to benthic predators. They reported that in the temperate climate of the Baltic Sea, cyanobacteria in the genera *Nodularia* and *Aphanizomenon* synthesize BMAA during extensive surface blooms (Jonasson et al., 2010). They also showed that higher concentrations of BMAA were detected in higher trophic level organisms in the Baltic Sea, including zooplankton, fish, and bivalve molluscs at times when cyanobacteria were abundant, suggesting the trophic transfer and biomagnification of the toxin and presenting a concern to human consumers (Jonasson et al., 2010).

A benthic consumer from the Chesapeake Bay that has the potential for BMAA accumulation is the iconic blue crab, *Callinectes sapidus*. Concern for the bioaccumulation of

BMAA in this commercially important species arose after a study performed by Field et al. (2013) examined three patients who developed ALS around the same time while living near each other in Annapolis, MD. One commonality between the patients was that they frequently consumed blue crabs. After testing three blue crabs at a local fish market, BMAA was identified, and the presence of BMAA in Chesapeake Bay food webs was confirmed (Field et al., 2013).

In this thesis, the growth of three commonly occurring cyanobacterial species was examined —*Microcystis aeruginosa*, *Synechococcus bacillaris*, and *Nostoc* sp. — which had previously been found to produce BMAA in culture systems, to determine the environmental conditions that promote BMAA synthesis by these organisms. These species were first grown under nutrient replete conditions to determine whether BMAA production varied over their growth cycle. Subsequently, *Microcystis aeruginosa* was grown under nitrogen and phosphorus deplete conditions to determine whether nutrient stress triggered BMAA production, as had previously been shown for this species (Downing et al., 2011). BMAA concentrations were also measured in natural phytoplankton assemblages dominated by cyanobacteria. Finally, BMAA accumulation into the muscle, hepatopancreas, and stomach tissues of blue crabs collected from three locations in the Chesapeake Bay known to be in proximity to cyanobacterial blooms was examined to observe BMAA biomagnification within the Chesapeake Bay food web.

# CHAPTER II
# CYANOBACTERIAL GROWTH AND THE POTENTIAL PRODUCTION OF BMAA

## Introduction

*Controls on cyanobacterial synthesis of free and protein-bound BMAA*

Cyanobacterial species occur in freshwater, estuarine, and marine environments, are pervasive worldwide, and their abundance and the incidence of blooms appears to be increasing due to warming water temperatures and anthropogenic eutrophication (Brand et al., 2010; Carmichael, 2013; Paerl and Otten, 2013). Nutrients including nitrogen and phosphorus that enter coastal environments are essential to cyanobacteria growth, but too much of these nutrients can lead to harmful algal blooms that can be deadly to aquatic organisms (Brand et al., 2010; Carmichael, 2013). These blooms can also be a problem to society, as they clog wastewater treatment facilities, create non-potable drinking water, and produce irritating smells (Shin et al., 2009). Additionally, many cyanobacteria can produce toxins that are harmful to both animals and humans, affecting the skin and mucous membranes, the digestive system and the nervous system (Carmichael, 1992; Carmichael, 1994; Chorus and Bartram, 1999; Sivonen, 2009).

Cyanobacteria are photoautotrophs, requiring dissolved inorganic carbon, the macronutrients nitrogen and phosphorus, water, and light to survive (Chorus and Bartram, 1999). Because they are bacteria, higher water temperatures and greater nutrient concentrations generally result in faster growth rates (Giannuzzi, 2018). Cyanobacteria growing naturally experience similar phases of growth to species grown in culture, beginning with the lag phase, where there is little reproduction and acclimation to environmental conditions occurs, followed by the exponential phase, during which cells double until the

environment has been depleted of nutrients or something else limits their growth (Giannuzzi, 2018). The stationary growth phase is characterized by no net growth, with subsequent population decline during the death phase, characterized by dying cyanobacterial cells due to environmental conditions unconducive to growth (Giannuzzi, 2018).

The production of secondary metabolites, including BMAA, was examined over the complete growth cycle (lag phase, exponential/log phase, stationary phase, and death) of three cultured cyanobacterial species (Li et al., 2014). The lag phase occurs after inoculation and dilution of cultured cells in fresh media, and typically not much growth is seen during this phase (Navarro Llorens et al., 2010). This phase is analogous to the period of time before a bloom initiates and is a period during which cells adapt to the culture conditions and repair intracellular damages that may have occurred before transfer into fresh media (Rolfe et al., 2012). The exponential phase represents the stage of rapid cell division and is analogous to bloom initiation and development. Growth continues at a constant rate if optimal growth conditions persist and nutrient concentrations are not limiting (Rolfe et al., 2012). Stationary phase occurs when cell division slows, most commonly due to the exhaustion of nutrients, light limitation or space (Navarro Llorens et al., 2010). This stage is analogous to the peak of a bloom in the environment. Due to the accumulation of waste products during the stationary phase, in addition to nutrient depletion, cultures subsequently enter the death phase, where cell numbers begin to decline (Navarro Llorens et al., 2010).

Optimal growth requirements vary among species of cyanobacteria (Berg and Sutula, 2015). Nitrogen and phosphorus are the most important macronutrients for cyanobacterial growth, and growth is diminished if either is lacking (Gerloff and Skoog, 1957; Berg and Sutula, 2015). Trace elements, including iron, molybdenum, copper, and manganese, can also

limit cyanobacterial growth if concentrations are too low (Rueter and Peterson, 1987). As photoautotrophs, cyanobacteria require light to perform photosynthesis. Growth rates tend to increase with increasing light levels and temperature (Spencer et al., 2011). Although there are differences between species, irradiance levels between 350-950 μmol photons $m^{-2}$ $s^{-1}$ yield the highest photosynthetic activity (Spencer et al., 2011; Berg and Sutula, 2015). Cyanobacteria typically thrive at warmer temperatures between 20-30 °C or higher, and over a range of salinities, depending on the species (Spencer et al., 2011; Berg and Sutula, 2015). In many coastal environments, it has been observed that toxin-producing cyanobacteria thrive in mesohaline (5-15 ppt) waters (Berg and Sutula, 2015). Because nearly all cyanobacteria were shown to produce BMAA in a previous study (Cox et al., 2005), I examined BMAA production by three cyanobacteria taxa previously shown to produce BMAA under nutrient-replete conditions during each phase of their growth.

When nitrogen and/or phosphorus are limiting, the production of photosynthetic pigments in cyanobacteria is restricted and photosynthetic activity decreases, although the cells can remain viable (Aguirre von Wobeser et al., 2011). For example, Sauer et al. (2001) starved *Synechococcus* sp. of nitrogen by transferring exponentially growing cells into nitrogen deplete media, which decreased photosynthetic activity and caused cell bleaching, though the cells remained alive. Aguirre von Wobeser et al. (2011) grew *Synechocystis* sp. under nitrate deplete conditions and found that with nitrogen and light limitation, *Synechocystic* sp. displayed low photosynthetic potential as well as significant changes in gene expression (Aguirre von Wobeser et al., 2011). In a study performed by Downing et al. (2011), nitrogen starvation of nutrient replete cells yielded an increase in free cellular BMAA by *Microcystis* sp. Furthermore, the addition of nitrate ($NO_3^-$) and ammonium ($NH_4^+$) to

starved *Microcystis* cells immediately decreased free cellular BMAA concentrations (Downing et al., 2011). These results suggest that BMAA is generated to promote cell survival during periods of nitrogen starvation (Downing et al., 2011).

The hypotheses that BMAA synthesis by three cyanobacterial species—*Microcystis aeruginosa, Synechococcus bacillaris,* and *Nostoc* sp. — varies over a growth cycle and that nitrogen limitation increases BMAA production by *Microcystis aeruginosa* were tested. It was hypothesized that cyanobacteria would produce BMAA at different rates during different parts of their growth cycle as cell physiology and culture conditions change. It was also hypothesized that *Microcystis aeruginosa* produce more BMAA under nutrient depleted conditions and that BMAA will occur in both protein-bound and freely cellular forms, but the protein-bound fraction would be greater. Findings from this study, will allow for a better understanding of when and under what conditions BMAA is produced in the environment.

**Methods**

*Microcystis aeruginosa*, *Synechococcus bacillaris,* and *Nostoc* sp. were grown under nutrient replete conditions to determine whether BMAA production varies as a result of population growth stage (e.g., lag phase, exponential phase or stationary phase growth). *Microcystis aeruginosa* was then grown in nutrient depleted media to determine whether the production of BMAA was enhanced under nutrient stress. Cellular distributions of BMAA as either protein-bound or freely soluble intracellular forms were measured via tandem HPLC-MS/MS to confirm its presence and that of structurally similar isomers, such as diaminobutyric acid (DAB).

The *Microcystis aeruginosa* culture used in this study was provided by Dianne Greenfield at the University of South Carolina (USC) and the *Synechococcus bacillaris* and *Nostoc* sp. species were purchased from the National Center for Marine Algae and Microbiota (NCMA) (CCMP1333 and CCMP2511, respectively). The *Microcystis aeruginosa* was originally collected from a retention pond at USC and grown in f/20 media at 24-26 (°C), a salinity of 15-17, and under light conditions of 22.74 µE $m^{-2}$ $s^{-1}$ in one-liter glass bottles (Table 3). The *Synechococcus bacillaris* and *Nostoc* sp., originally obtained from Long Island Sound near Milford, CT and Kaneohe Bay, Oahu, HI respectively, were grown in L1-Si media at 24-26 (°C), a salinity of 30-32, and under light conditions of 22.74 µE $m^{-2}$ $s^{-1}$ in one-liter glass bottles (Table 3). A parent culture of each species was split and diluted with fresh media into fifteen, 500 mL bottles to be sacrificed at five different time points corresponding to different stages of their growth: lag phase, early exponential phase, late exponential phase, stationary phase, and late stationary phase. Growth curves were constructed by measuring the *in vivo* fluorescence of cultures over time. Triplicate culture bottles were sacrificed at each time point, and samples were collected to measure free and protein-bound BMAA concentrations, particulate organic carbon and nitrogen (POC/PON), cyanobacteria cell number, and heterotrophic bacteria cell numbers in each culture.

**Table 3.** Origin of cyanobacteria used and culture conditions of each species. Light conditions were constant at 22.74 μE $m^{-2}$ $s^{-1}$ over a 24-hour period for each culture.

| **Species** | **Origin** | **Medium** | **Temperature (°C)** | **Salinity** |
|---|---|---|---|---|
| *M. aeruginosa* | Brackish coastal ponds, USC | f/2 with f/20 nutrients | 24-26 | 15-17 |
| *S. bacillaris* | Long Island Sound, Milford, CT | L1-Si | 24-26 | 30-32 |
| *Nostoc* sp. | Kaneohe Bay, Oahu, HI | L1-Si | 24-26 | 30-32 |

In addition, *Microcystis aeruginosa* was also grown semi-continuously under nutrient limited conditions. For these treatments, triplicate one-liter cultures were maintained using artificial seawater with a salinity of 17 ppt and f/200 nutrients, with ten times less $NaNO_3$ and one hundred times less $NaH_2PO_4$ than prescribed (Guillard and Ryther, 1962; Guillard, 1975). The final concentrations in the media were 8.8 μM $NaNO_3$ and 0.04 μM $NaH_2PO_4$. Cells were grown until exponential phase was reached (approximately 14 days of growth), and were then switched to a semi-continuous culture mode. This was done by calculating their growth rate (GR) approximately every two days utilizing raw *in vivo* fluorescence values (IVF) obtained via fluorometer and time (t) in the following equation:

Equation 1: $GR = \frac{\ln (FlT2 / FlT1)}{t}$

At each dilution, a specific volume of culture was removed and replaced with fresh f/200 media to maintain growth in exponential phase, using the following equation: Volume = GR * culture volume. The culture volume that was removed was then filtered, and the filtrate later analyzed to ensure that nitrogen and phosphorus were being exhausted by the cyanobacteria during growth.

For both the nutrient replete and nutrient limited experiments, particulate organic carbon (POC) and nitrogen (PON) samples were collected to determine the relationships between toxin production and cellular C and N. This was done by filtering 25 mL of culture onto combusted 25 mm GF/F filters, to which 1-2 drops of 4 M HCl were applied to remove any residual inorganic carbon from the filter. The filters were then placed in a 60 °C drying oven for approximately 24 hours, and then sent to the Water Quality Analysis Laboratory in Norfolk, VA to determine POC and PON concentrations (USEPA, 1997).

Cyanobacteria cell count samples were prepared by collecting 10 mL of culture and adding 2-3 drops of Lugol's fixative for the preservation of cells. Cyanobacteria cell counts for *Microcystis aeruginosa, Synechococcus bacillaris,* and *Nostoc* sp. were determined by placing preserved samples in a hemocytometer for microscopic enumeration using an Olympus CKX41 epifluorescent microscope on 10x-20x magnification based on methods of Humphries and Widjaja (1979). Samples for enumeration of bacterial cells were prepared by collecting 2 mL from each culture and adding 200 μL of 10% glutaraldehyde for preservation. The 2 mL of preserved culture was filtered on a fritted glass filtration base onto a 0.2 μm pore size 25 mm diameter black filter. The bacterial cells were stained with one or two drops of 4′,6-diamidino-2-phenylindole (DAPI) with Vectashield mounting medium on the center of the filter in preparation for counting (Porter and Feig, 1980; Noble and Furhman,1998). Bacterial cells were enumerated using an Olympus BX-50 epifluorescent microscope at 2000x magnification using an excitation wavelength of 330-385 nm, and an emission of 420 nm. Cell counts and bacteria counts were performed in the same manner for the nutrient deplete *Microcystis aeruginosa* samples.

*High performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) of cellular extracts*

While many other analytical methods have been used in previous studies (Cox et al., 2003; Cox et al., 2005; Jonasson et al., 2010; Spácil et al., 2010), for this study the concentration of BMAA was determined by HPLC-MS/MS in nutrient replete and nutrient deplete cyanobacterial cultures. An Agilent 1290 infinity binary pump LC was interfaced to a bonded silica C18 Dionex Acclaim Polar advantage rapid separation liquid chromatography (RSLC) column (2.2 µm, 120 A X 150 mm) for chromatographic separation. A guard column with a 5 µm particle size having the same column packing as the analytical column was used. Samples were injected on the column using an Agilent auto sampler at 10 µl per injection and with a mobile phase flow rate set at 0.3 mL $min^{-1}$. The gradient used for mobile phase A included the ion-pairing reagent 4′,6-diamidino-2-phenylindole (HFBA) (0.4% of phase by volume), formic acid (0.02% of phase) and HPLC-MS grade water. Mobile phase B included formic acid (0.1% of phase) in acetonitrile. The gradient program, modified from Piraud et al. (2005), utilized 100% mobile phase A from 0 to 1 minutes, 85% A from 1 to 6 minutes, 85% to 75% from 6 to 9 minutes, and 75% from 9 to 15 minutes. Then, from 15 to 16 minutes the gradient was set to an isocratic state of 100% mobile phase A, and finally from 16 to 31 minutes, 100% mobile phase A was maintained. All amino acids were observed over the first 16 minutes of each sample run. From 16 to 31 minutes of a sample run, the column was flushed with a 100% mobile phase A to remove impurities and restore the ion pairing reagent to the analytical column before analysis of the next sample.

The HPLC was integrated with a Thermo Scientific Orbitrap XL mass spectrometer via an ESI interface for structural analysis. Data was acquired and processed in Xcalibur

software (Thermo Scientific, Rockford, IL) at a scan range of *m/z* 50-300 using positive ion mode. The collision energy was kept constant through the entire run at 35 V with CID activation in the primary scan and a scan cycle (resolution) of 30000. The ESI source parameters included a capillary temperature of 275 °C, a spray voltage of 3.5 V, and a capillary voltage of 2.5 V. All ESI source parameters were tuned and maximized before sample runs and the ESI source utilized nitrogen as the carrier gas.

*Amino acid calibration and identification*

Given the low concentrations of BMAA expected in cultures, a series of calibrations were performed to establish minimum detection limits and response. Amino acid concentrations were quantified using an internal standard/external standard paired set to provide coverage of the major functional groups of the amino acids being determined. This method also allowed for the quantification of other common amino acids, such as leucine, isoleucine, and glutamate. An individual calibration curve was constructed for BMAA and DAB and was normalized to the internal standard D-2,4- Diaminobutyric -2,3,3,4,4 $d_5$ acid 2HCl ($D_3$AB). The final sample concentrations were determined based on the ratio of amino acid peak area to the internal standard peak area, using the following formulas:

$$(1)\quad PA\left(\frac{\text{amino acid}}{\text{ITSD}}\right) = m*\left(\frac{\text{concentration of Amino acid}}{\text{Concentration of ITSD}}\right)+b$$

$$(2)\quad AA_{conc.} = \frac{(PA-b)}{m} * \text{concentration of ITSD}$$

*PA* stands for the peak area of the selected amino acid divided by the peak area of the corresponding internal standard; *m* is the slope of the linear curve, while *b* is the *y*-intercept of

the linear calibration curve. "*AAconc"* is defined as the final concentration of an amino acid in each sample. ITSD is defined as the internal standard.

All samples were scanned for both the full mass spectrum, from 50 *m/z* to 500 *m/z*, and the daughter products ($MS^2$ spectrum) using Xcalibur software. Peaks in samples were identified based on their MS retention times and $MS^2$ daughter products. Primary ion identifiers of each amino acid were identified using values of M+H or molecular weight plus one for a hydrogen ion. This information was obtained from a series of injected standards, as well as literature values for MS and $MS^2$ (Faassen et al., 2012). Amino acid quantification was based on mass abundances (as peak area) of each identified amino acid compared to the internal standard selected for that amino acid. BMAA was detected by the transitions of *m/z* 119.1 (M+H) to *m/z* 102.1 ($MS^2$) and DAB detected by the transitions *m/z* 119.1 (M+H) to *m/z* 101 ($MS^2$) (Faassen et al., 2012).

*BMAA analysis in cyanobacteria cultures*

Freely soluble BMAA samples were prepared via freeze-thaw extraction (Houpert et al., 1976). Cells of the three cyanobacteria species sacrificed in late exponential phase, stationary phase, and late stationary phase of growth were pelletized by centrifugation (approximately 10 μL). To extract the cell contents, HPLC-MS grade methanol was added to each pellet in 1.5 mL Eppendorf tubes. The solution within each tube was then sonicated for 30 seconds at 10-15 pulses. The tubes were then immediately placed in liquid nitrogen for five minutes to assist lysis of cells, and then subsequently warmed at room temperature for five minutes. Tubes were then placed in a room temperature water bath for fifteen minutes to thaw the extracts. They were then centrifuged for ten minutes on a setting of 8000 times/min to separate the BMAA extracted in methanol from the cellular materials. The entire process

was repeated twice for a total of three freeze-thaw cycles. Tubes were stored at -80 °C until the day of extraction.

On the day of analysis, pellets from the Eppendorf tubes were transferred to micro-insert tubes, and methanol and internal standard D-2,4- Diaminobutyric -2,3,3,4,4 $d_5$ acid 2HCl ($D_3$AB) were added. Different concentrations of $D_3$AB were added to each sample based on proposed BMAA concentration in the cell pellets. From the lowest proposed BMAA concentration to highest concentration, 1.7 ng, 2.52 ng, or 3.40 ng were added respectively. Next, samples were dissolved in 200 µL of the ion-pairing reagent HFBA (0.4% of phase by volume), formic acid (0.02% of phase) and HPLC-MS grade water, and were stored at -80 °C until the day of analysis.

Protein-bound BMAA samples were extracted using acid hydrolysis (Kaiser and Benner, 2005). Cell pellets (approx. 10 µL) were added to 4 mL amber vials, and 1000 µL of 6 M HCl was added. The vials were immediately flushed with $N_2$ and sealed off with Teflon tape. The vials were heated to 110 °C for approximately 20 hours. Next, approximately 1000 µL of the hydrolysate was pipetted into 2 mL amber vials to be placed on the drying stand to completely evaporate all HCl with nitrogen. After drying, 20 µL of water was added and evaporated to ensure all acid was gone. This step was then repeated a second time. Again, 1.7 ng, 2.52 ng, and 3.40 ng of $D_3$AB were added to the lowest, medium, and highest proposed BMAA concentrations and were then evaporated. The samples were then stored at -80 °C prior to analysis. On the day of analysis, the samples were re-dissolved in 200 µL of the 0.1% aqueous HFBA.

Extraction of free and protein-bound BMAA from nutrient deplete cyanobacteria samples were prepared using the same methodology as the nutrient replete samples. Prior to

analysis, $D_3$AB was added to the hydrolyzed samples as an internal standard, which was then evaporated. On the day of analysis, the samples were re-dissolved in 200 μL of the ion-pairing reagent HFBA.

Three types of controls were included among the hydrolyzed samples. The first sample was a blank that was spiked with 90 μg/mL of BMAA standard. The second was a pellet of *M. aeruginosa* that was spiked with 90 μg/mL of BMAA standard, representing the "low" spike concentration. The final sample was a pellet of *M. aeruginosa* that was spiked with 900 μg/mL of BMAA standard, representing the "high" spike concentration.

*Minimum cellular detection limits*

The minimum cellular detection limits for nutrient replete cyanobacteria samples were calculated by utilizing the instrumental limit of detection of 106 pg and the fraction of hydrolyzed sample analyzed following injection. To detect BMAA in these cells, there would have to have been a concentration greater than 0.02 fg/cell or 20 pg/ $10^6$ cells (bloom conditions). Utilizing the lowest instrumental detection limit for the nutrient deplete cells of 25-100 pg, there would have to have been a BMAA concentration greater than 0.005 fg/cell or 5 pg/ $10^6$ cells. The instrumental detection limit was lowered from 106 pg to 25-100pg between nutrient replete and nutrient deplete cells to increase the possibility of BMAA detection at lower concentrations in nutrient deplete cells.

## Results

### *Cyanobacterial growth under nutrient replete and deplete conditions*

*Microcystis aeruginosa* was grown for a total of 37 days to complete a growth cycle. This species completed its lag phase after approximately 5 days, and then grew in exponential phase for the next 25 days (Figure 7). The *M. aeruginosa* stationary phase lasted from approximately day 30 to 35, after which it reached its late stationary phase until its final sampling (Figure 7).

*Synechococcus bacillaris* was grown for a total of 32 days, and the first sampling took place on day 8, when it completed its lag phase (Figure 7). Its exponential phase lasted from day 8 to 15, where it then remained in stationary phase until peaking once more at day 29. The *S. bacillaris* biomass then decreased from its highest value at day 29 back down to the biomass seen at its stationary phase (Figure 7).

*Nostoc* sp. was also grown for a period of 32 days, during which its lag phase lasted for the first 7 days, followed by an exponential phase ranging from day 11 to approximately day 21 (Figure 7). This species then reached its stationary phase at day 22, where it remained until its final sampling during the late stationary phase at day 32 (Figure 7).

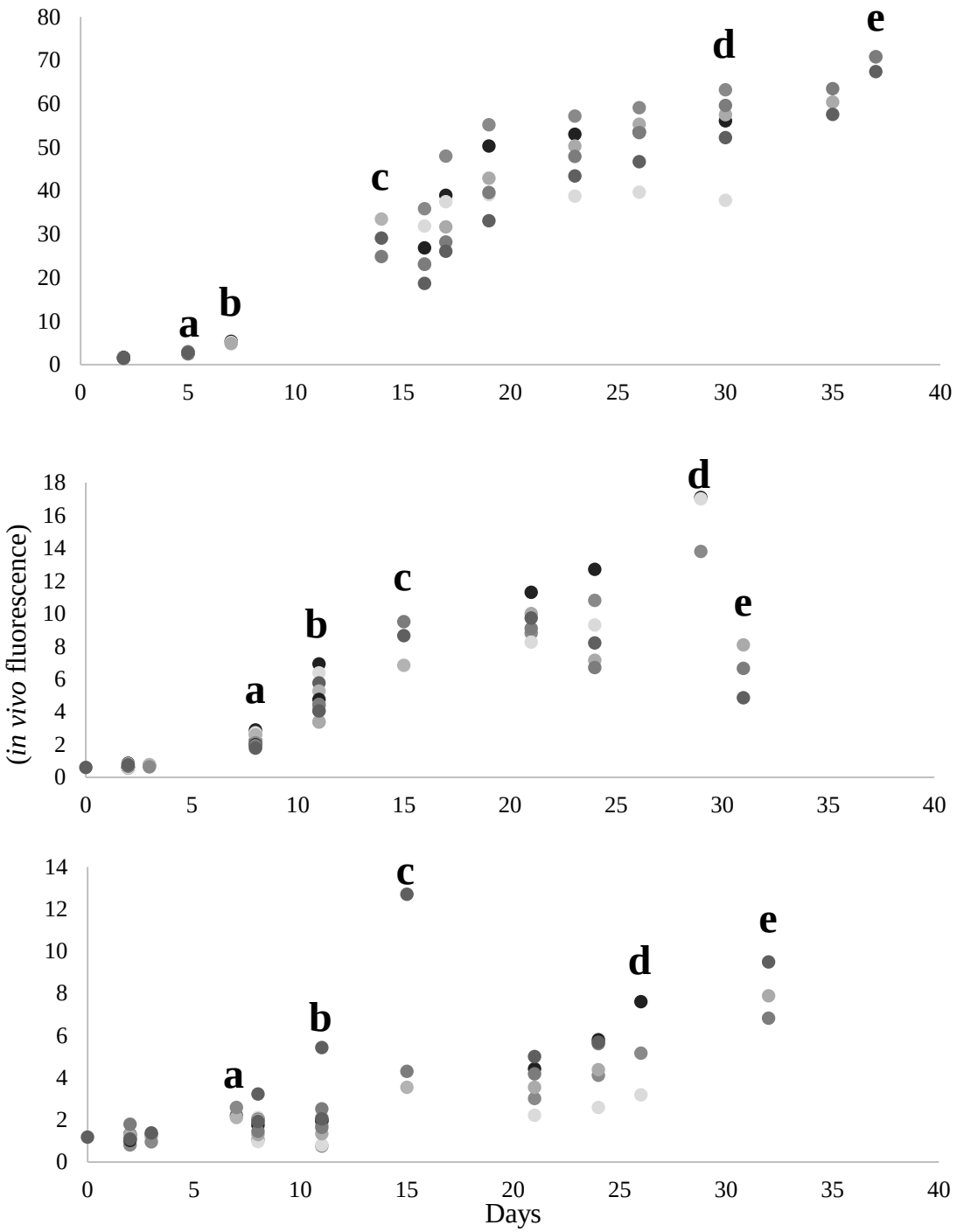


**Figure 7.** Growth curves for *Microcystis aeruginosa* (upper panel), *Synechococcus bacillaris* (middle panel), and *Nostoc* sp. (lower panel). Different shades of color represent triplicate cultures. Each species was placed into five groups of three bottles, to be sacrificed at each of the following time points along their individual growth curves: lag phase (a), early exponential phase (b), late exponential phase (c), stationary phase (d), and late stationary phase (e). *Microcystis aeruginosa* sacrificial time points correspond to days 5, 8, 14, 30, and 37. *Synechococcus bacillaris* sacrificial time points correspond to days 8, 12, 15, 29, and 32. *Nostoc* sp. sacrificial time points correspond to days 7, 11, 15, 26, and 32.

Cultures of *M. aeruginosa* were grown semi-continuously under low-nutrient conditions and the average growth rate estimated using *in vivo* fluorescence values (IVF), a proxy for chlorophyll concentration, was plotted for the entire acclimation period (Figure 8). Semi-continuous growth in the exponential phase was started on day 14 (represented by the dotted line) (Figure 8). After 3 generations of acclimation to low nutrient conditions (growth rate = 0.051 cells/day) and no additional fresh media, triplicate cultures were sacrificed over time. Average $NO_3^-$ + $NO_2^-$ ($NO_x$) concentrations were at or near the limit of analytical detection (between 0.09 and 0.4 μM) when each semi-continuous dilution was made (Figure 9). Figure 9 shows $NO_x$ (μM) concentrations after switching to semi-continuous growth and demonstrates that cells were maintained at very low external $NO_x$ (average of 1.19 μM) concentrations, near the limit of analytical detection.

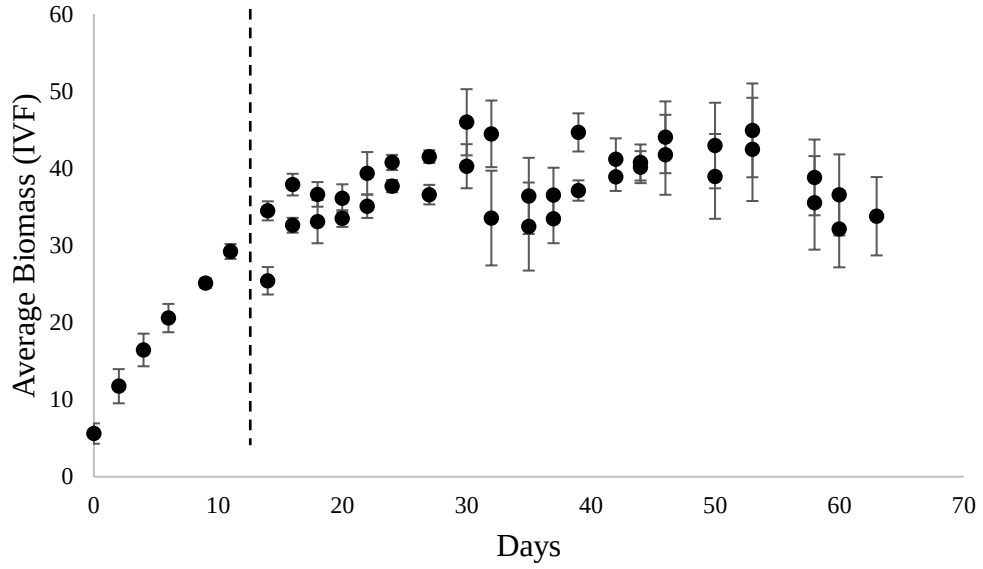


**Figure 8.** Growth of *Microcystis aeruginosa* to exponential phase (day 14) and subsequent semi-continuous growth for 3 generations, allowing for acclimation to low nutrient media (days 14-63). Error bars represent the standard error within triplicate samples of average biomass from each day the values were obtained.

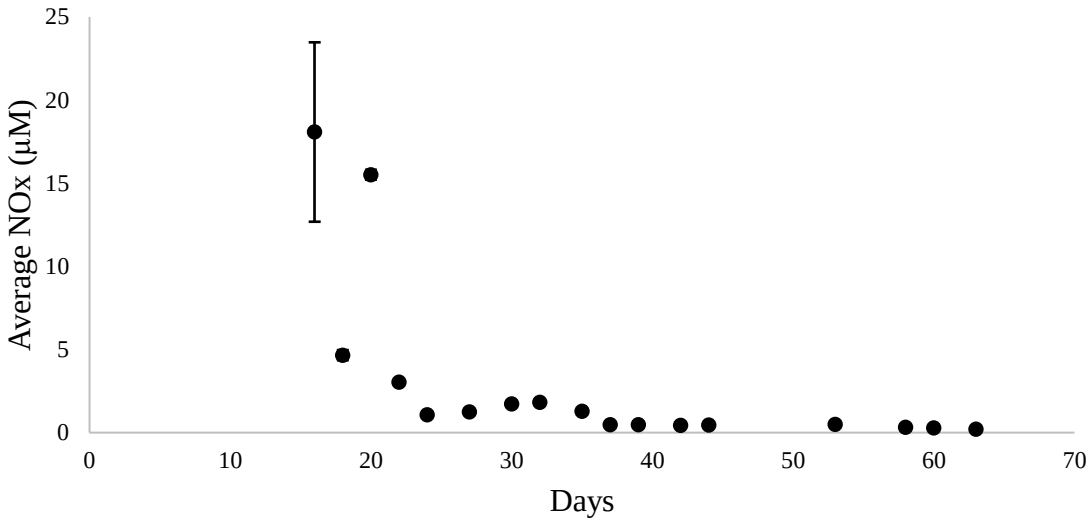


**Figure 9.** Average dissolved $NO_x$ (μM) concentrations in the cultures following each semi-continuous dilution. Error bars represent the standard error within triplicate samples of $NO_x$ (μM) concentrations from each day the concentrations were obtained, and those not shown are smaller than the symbol area.

Particulate organic nitrogen and carbon concentrations steadily increased with time for *Microcystis aeruginosa* and *Nostoc sp* (Table 4). This pattern followed that of *in vivo* fluorescence (Figure 7). No decrease in PON and POC was seen in the final stages of their growth (Table 4). The PON and POC concentrations for *Synechococcus bacillaris* increase through late exponential and stationary phase, and then began to decrease in late stationary phase (Table 4).

For the nutrient limited *M. aeruginosa* cultures, the PON increased slightly from 0.48 to 0.62 mg/L after stopping the dilutions, and then remained at 0.63 for the remainder of the starvation period (Table 4). The concentrations of POC did not significantly differ between any of the time points, remaining between 8.2 and 9.4 mg/L (Table 4).

The C/N ratio for *M. aeruginosa* began at 16.9 during the lag phase, and then increased to 24.38 by the early exponential phase (Figure 10). It then decreased to approximately 15 for the

late exponential and stationary phases, and then dropped again to 6.1 at the late stationary phase (Figure 10). The ratio for *S. bacillaris* decreased only from 5.5 to 3.9 throughout the entire growth cycle (Figure 10). Similarly, the C/N ratio for *Nostoc* sp. ranged from 4.2 to 5.3 throughout the growth cycle (Figure 10). Finally, the C/N ratio for nutrient limited *M. aeruginosa* began at 17.26 during day 2 of complete nutrient starvation, and then decreased to 12.99 by day 8 (Figure 10).

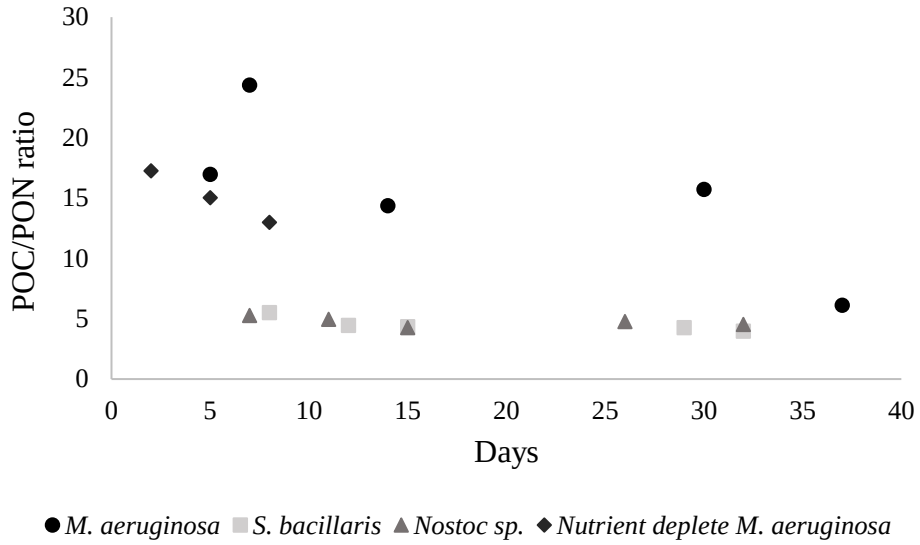


**Figure 10.** C/N ratios vs days for *Microcystis aeruginosa, Synechococcus bacillaris, Nostoc* sp., and nutrient limited *Microcystis aeruginosa.* The values for *M. aeruginosa, S. bacillaris,* and *Nostoc* sp. were obtained on days corresponding to the growth curve phases. Values for nutrient limited *M. aeruginosa* were obtained on days corresponding to the sacrifice of triplicate cultures after acclimation to low nutrients.

Cellular carbon concentrations were calculated for each culture sample collected, and represent the maximum carbon concentrations possible, as they include both cyanobacterial and bacterial carbon. For *M. aeruginosa*, organic carbon per cell was $7.87*10^{-6}$ μg/cell during lag phase, but then dropped to approximately $3*10^{-6}$ μg/cell during the early and late exponential phases (Figure 11). It then increased again to $7.79*10^{-6}$ μg/cell during the stationary phase, and then decreased to $2.12*10^{-6}$ μg/cell at the late stationary phase (Figure 11). For *S. bacillaris*, POC/cell was calculated during late exponential, stationary, and late stationary growth phases, as cell counts were not available for the first two growth phases (Figure 11). POC/cell was between $1.09*10^{-5}$ μg/cell and $1.97*10^{-5}$ μg/cell (Figure 11). For *Nostoc* sp., the POC/cell began at $7.39*10^{-6}$ μg/cell in the late exponential phase, but then increased to $1.60*10^{-5}$ μg/cell during stationary phase growth (Figure 11). It decreased slightly to $1.46*10^{-5}$ μg/cell in late stationary phase (Figure 11). For nutrient limited *M. aeruginosa*, POC/cell was approximately $5*10^{-6}$ μg/cell at days 2 and 5, and then decreased to $4.45*10^{-6}$ μg/cell by day 8 (Figure 12).

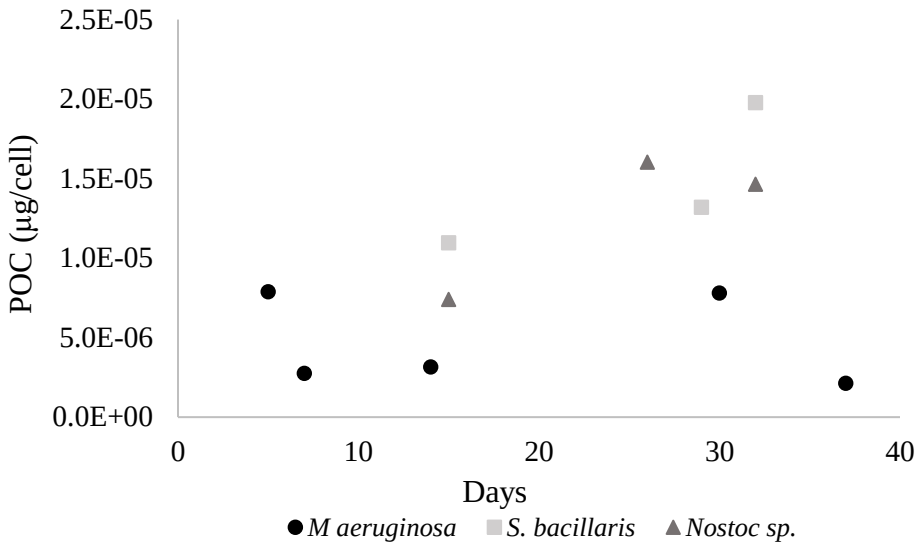


**Figure 11.** POC/cell concentrations vs. days for *M. aeruginosa*, *S. bacillaris*, and *Nostoc* sp. The values for *M. aeruginosa* were obtained on days corresponding to the growth curve phases. Values for *S. bacillaris*, and *Nostoc* sp. correspond to the late exponential, stationary, and late stationary growth phases, as cell counts were not available for the lag phase and early exponential phase.

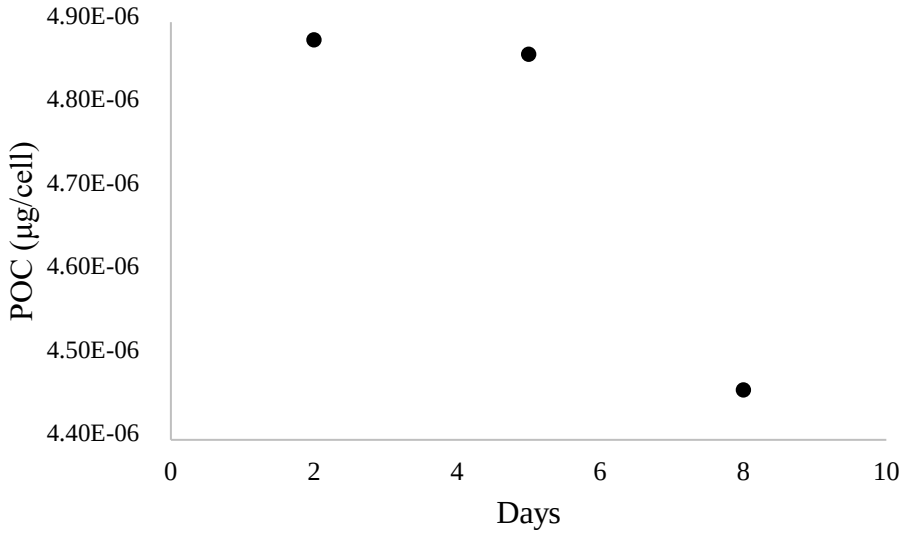


**Figure 12.** POC/cell concentrations vs. days for nutrient limited *M. aeruginosa*. Values were obtained on days corresponding to the sacrifice of triplicate cultures after acclimation to low nutrients.

**Table 4.** PON (mg/L), maximum POC (mg/L), C/N ratio, and maximum POC (µg)/cell for *M. aeruginosa, S. bacillaris, Nostoc* sp., and nutrient limited *M. aeruginosa.* Carbon concentrations are the maximum concentrations possible as they represent both cyanobacterial and bacterial carbon. The values for nutrient replete *M. aeruginosa* were obtained on days corresponding to the growth curve phases. Values for *S. bacillaris,* and *Nostoc* sp. correspond to the late exponential, stationary, and late stationary growth phases, as cell counts were not available for the lag phase and early exponential phase. The values for nutrient deplete *M. aeruginosa* were obtained on days corresponding to the sacrifice of triplicate cultures after acclimation to low nutrients.

| | **Day of sacrifice** | **PON (mg/L)** | **POC (mg/L)** | **C/N ratio** | **POC (µg/cell)** |
|---|---|---|---|---|---|
| *M. aeruginosa* | | | | | |
| | 5 | 0.18 | 3.05 | 16.98 | $7.87*10^{-6}$ |
| | 7 | 0.11 | 2.79 | 24.38 | $2.75*10^{-6}$ |
| | 14 | 0.27 | 3.84 | 14.37 | $3.15*10^{-6}$ |
| | 30 | 0.42 | 6.57 | 15.73 | $7.79*10^{-6}$ |
| | 37 | 1.78 | 10.90 | 6.11 | $2.12*10^{-6}$ |
| *S. bacillaris* | | | | | |
| | 8 | 1.17 | 6.47 | 5.53 | Cell count N/A |
| | 12 | 3.04 | 13.59 | 4.47 | Cell count N/A |
| | 15 | 3.88 | 16.85 | 4.34 | $1.09*10^{-5}$ |
| | 29 | 8.22 | 35.15 | 4.28 | $1.31*10^{-5}$ |
| | 32 | 6.28 | 25.00 | 3.98 | $1.97*10^{-5}$ |
| *Nostoc sp.* | | | | | |
| | 7 | 1.44 | 7.60 | 5.29 | Cell count N/A |
| | 11 | 1.81 | 8.98 | 4.96 | Cell count N/A |
| | 15 | 4.48 | 19.07 | 4.26 | $7.39*10^{-6}$ |
| | 26 | 3.84 | 18.33 | 4.77 | $1.60*10^{-5}$ |
| | 32 | 5.03 | 22.78 | 4.53 | $1.46*10^{-5}$ |
| Nutrient deplete *M. aeruginosa* | | | | | |
| | 2 | 0.49 | 8.46 | 17.27 | $4.88*10^{-6}$ |
| | 5 | 0.62 | 9.38 | 15.04 | $4.86*10^{-6}$ |
| | 8 | 0.63 | 8.23 | 12.99 | $4.45*10^{-6}$ |

Bacteria counts were made for each cyanobacteria culture during late exponential, stationary, and late stationary phase growth to account for contaminating bacteria. For *M.*

*aeruginosa*, bacteria abundance increased by 3.0 $*10^5$ cells/mL from late exponential phase to late stationary phase (Table 5). The bacteria abundance in *S. bacillaris* cultures were the highest of all three species, increasing from about 1.0 $*10^6$ bacteria/mL at late exponential phase, to over 2.0 $*10^6$ bacteria/mL in stationary phase, and then rose again above 4.0 $*10^6$ in the late stationary phase (Table 5). For *Nostoc sp.*, bacterial numbers were about 8.5 $*10^5$ cells/mL in late exponential phase increasing to over 1.0 $*10^6$ cells/mL during the stationary phase, before decreasing during late stationary phase to 6.7 $*10^5$ cells/mL (Table 5). For the nutrient limited *M. aeruginosa*, bacterial abundance was constant at all time points (Table 5).

**Table 5.** Bacterial abundance in cultures of *M. aeruginosa, S. bacillaris, Nostoc* sp., and nutrient limited *M. aeruginosa.* Values for *M. aeruginosa, S. bacillaris,* and *Nostoc* sp. correspond to the late exponential, stationary, and late stationary growth phases, as cell counts were not available for the lag phase and early exponential phase. The values for nutrient deplete *M. aeruginosa* were obtained on days corresponding to the sacrifice of triplicate cultures after acclimation to low nutrients.

| | **Day of sacrifice** | **# Bacteria/mL** |
|---|---|---|
| *M. aeruginosa* | | |
| | 14 | $1.13 *10^5$ |
| | 30 | $1.58 *10^5$ |
| | 37 | $3.99 *10^5$ |
| *S. bacillaris* | | |
| | 15 | $1.09 *10^6$ |
| | 29 | $2.12 *10^6$ |
| | 32 | $4.07 *10^6$ |
| *Nostoc* sp. | | |
| | 15 | $8.49 *10^5$ |
| | 26 | $1.04 *10^6$ |
| | 32 | $6.74 *10^5$ |
| Nutrient deplete *M. aeruginosa* | | |
| | 2 | $2.63 *10^5 \pm 4.8*10^4$ |
| | 5 | $2.89 *10^5 \pm 217$ |
| | 8 | $1.72 *10^5 \pm 4.3*10^4$ |

*BMAA detection limits for cells*

For the nutrient replete cyanobacteria samples, the minimum detection limit was 106 pg (Figure 14). To detect any BMAA in these cells, there would have to have been a concentration greater than 0.02 fg/cell or 20 pg/ $10^6$ cells (bloom conditions). For the nutrient deplete samples, the minimum detection limit was between 25 and 100 pg (Figure 1-A seen in appendix section). To detect any BMAA in these cells, there would have to have been a concentration greater than 0.005 fg/cell or 5 pg/ $10^6$ cells.

Despite the very low minimum detection limits established, no free or protein-bound BMAA was observed in any culture sample analyzed from all three nutrient replete species tested. All samples were compared to the calibration curve which included both BMAA and DAB (Figure 15). BMAA was also absent in samples of nutrient limited *M. aeruginosa*. Samples were also compared to the respective calibration curve including both BMAA and DAB (Figure 16).

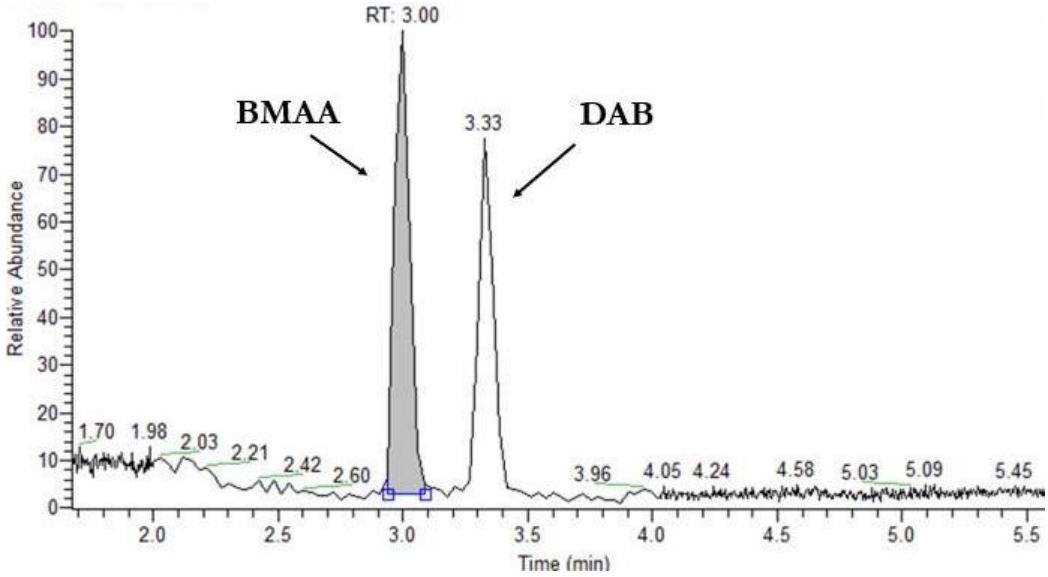


**Figure 13.** Partial chromatogram of standard peaks of BMAA and DAB from nutrient replete cyanobacteria sample, showing that both isomers are present in the standard samples.

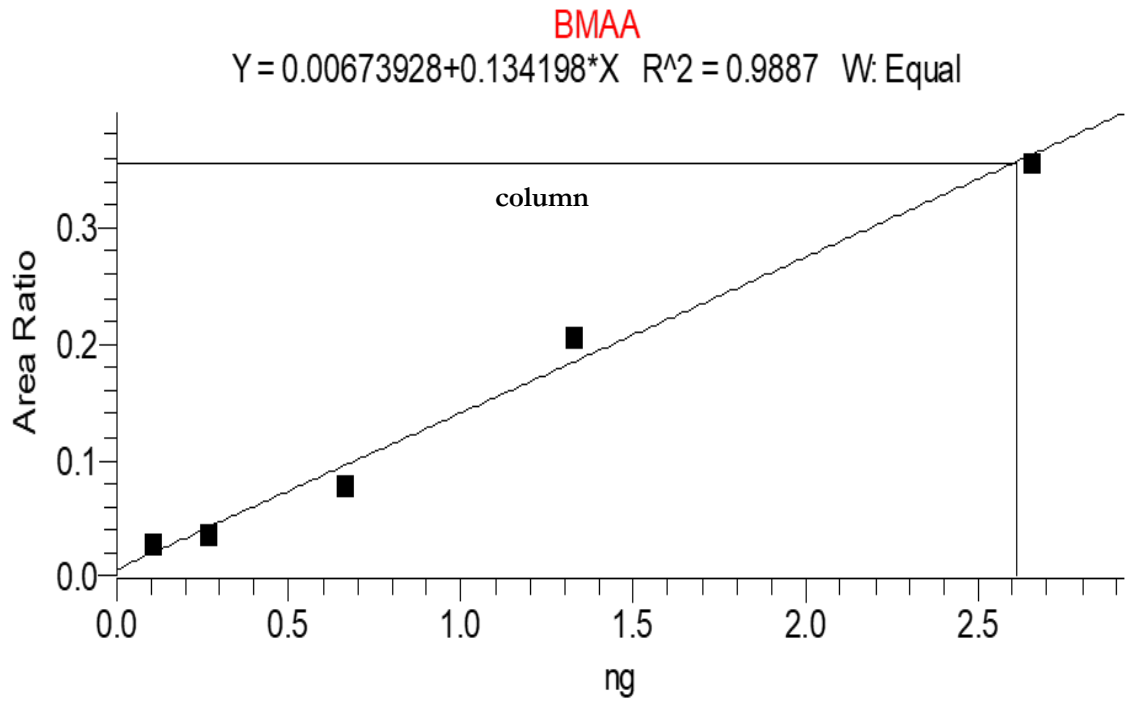


**Figure 14.** HPLC-MS/MS calibration curve for the nutrient replete cyanobacteria samples. The limit of detection was 106 pg per injection.

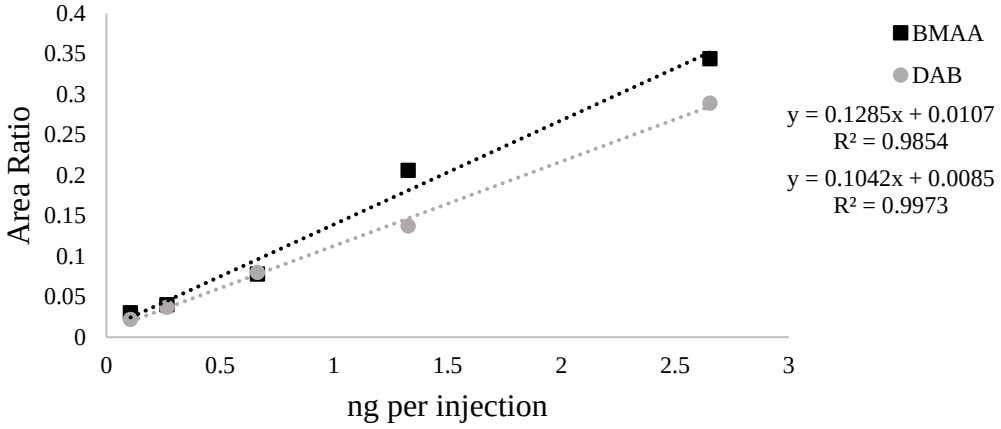


**Figure 15.** Calibration curve of BMAA and DAB for nutrient replete cyanobacteria, showing a limit of detection of 106 pg. Area ratio is the ratio of the external standard (DAB or BMAA) divided by internal standard ($D_3$AB).

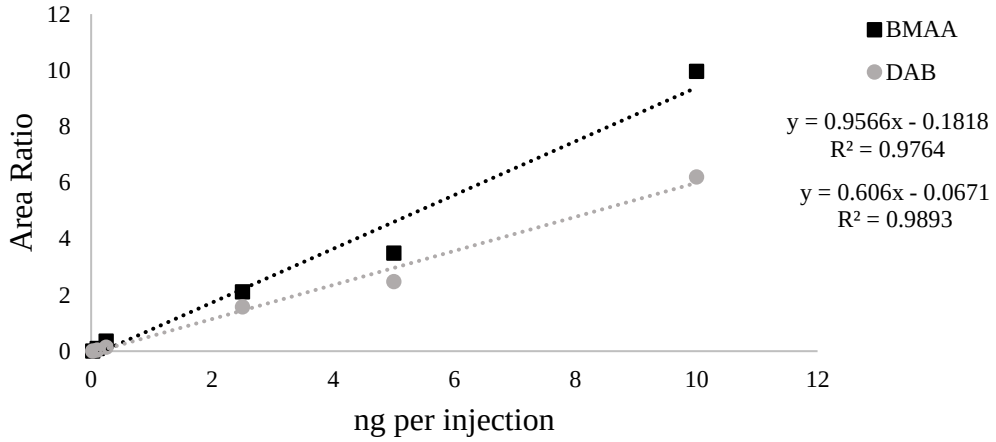


**Figure 16.** Calibration curve of BMAA and DAB for nutrient deplete *M. aeruginosa*, showing a limit of detection of 25-100 pg. Area ratio is the ratio of the external standard (DAB or BMAA) divided by internal standard ($D_3$AB).

*Sample analysis and detection*

To ensure that the absence of BMAA was not due to matrix effects, all samples analyzed were compared to BMAA-spiked *M. aeruginosa* pellets from the nutrient replete culture samples, that were previously determined to have no BMAA. The "high" spike contained 900 µg/mL of BMAA standard and the "low" spike contained 90 µg/mL of BMAA standard (Figure 17). Additionally, one blank sample was spiked with 90 µg/mL of BMAA (Figure 17). It is very clear after comparing the spiked samples and the nutrient deplete samples that there was no BMAA present in the latter, as there are no peaks occurring at retention times 3.00 and 3.33 minutes with an *m/z* of 119>101.

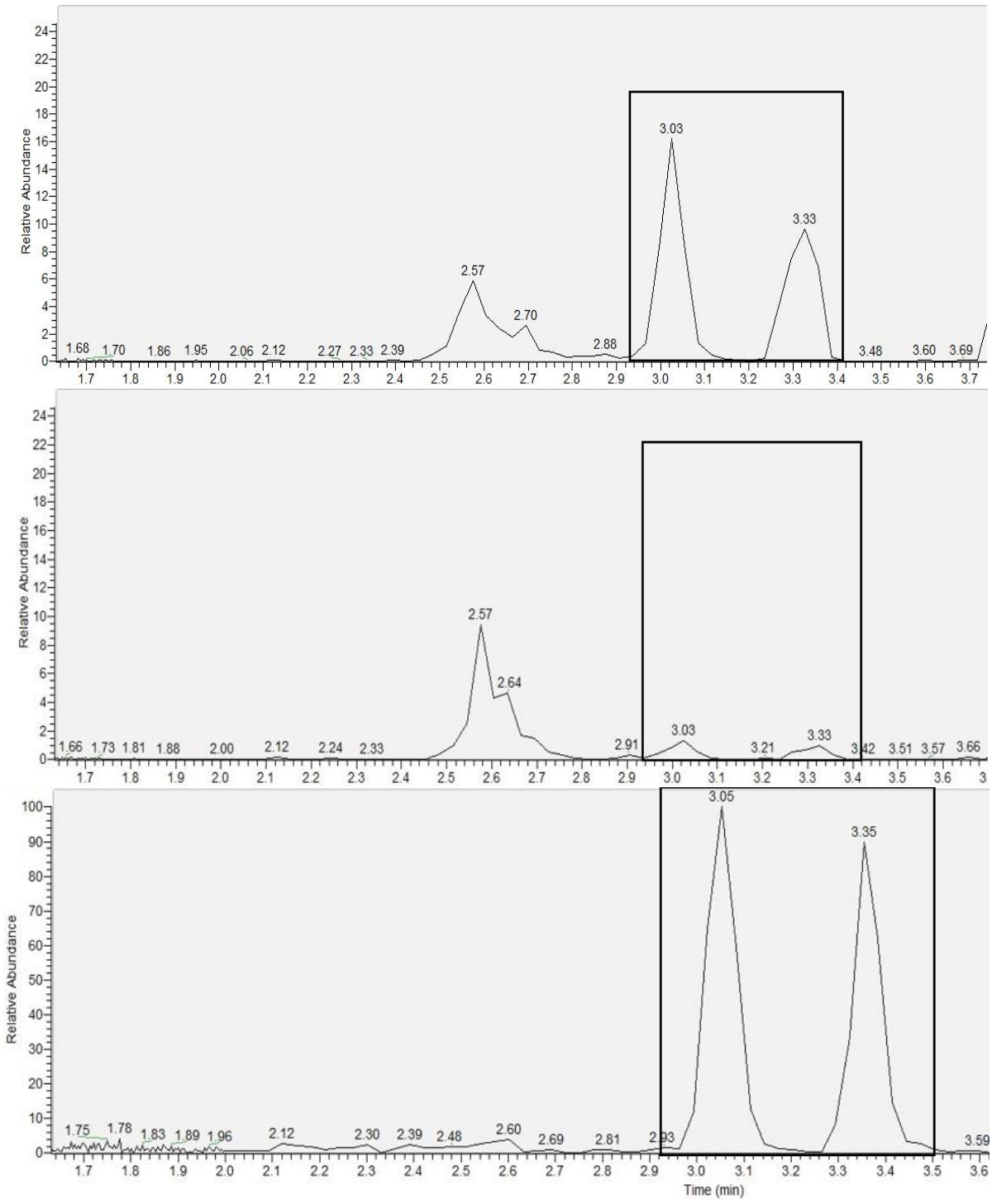


**Figure 17.** (Top): Partial chromatogram of *M. aeruginosa* nutrient replete pellet spiked with 900 µg/mL of BMAA standard, (middle panel) *M. aeruginosa* nutrient replete pellet spiked with 90 µg/mL of BMAA standard, and (bottom) blank sample spiked with 90 µg/mL of BMAA standard. The black square represents the retention time frame of a BMAA and DAB peak.

## Discussion

### *Nutrient constraints and growth stages*

Despite the detection of the putative neurotoxin BMAA in various cyanobacteria species in previous studies (Cox et al., 2005), BMAA was not detected at any growth phase among three species tested here. Possible reasons for this include the inhibition of cyanobacterial toxin production under laboratory conditions or the need for specific adverse environmental conditions to induce production, bacterial decomposition of BMAA, and its concentration in cells lower than the detection limit of 25-106 pg used in this study (Schatz et al., 2005; Harvey et al., 2006; Li et al., 2010; Downing et al., 2011; Liu et al., 2017).

One explanation for the absence of BMAA in cultures is that cyanobacteria may be less likely to produce it under laboratory conditions than in nature. The species involved in this study had been grown under controlled laboratory conditions for months before BMAA testing. Li et al. (2010) performed a study in which they cultured axenic strains of *Microcystis aeruginosa* and *Nostoc* sp., originally collected in China, and then subsequently grown in the lab. They reported that cultured *Nostoc* sp. produced BMAA in very low concentrations, but they were unable to detect BMAA in cultures of *M. aeruginosa* (Li et al., 2010). They speculated that BMAA production was hindered by cyanobacteria culture conditions (Li et al., 2010). In contrast to the conclusion by Cox et al. (2005) that nearly all cyanobacteria produce BMAA, there has not been a consistent cyanobacterial strain that produces BMAA under laboratory conditions (Monteiro et al., 2016). Cox et al. (2005) concluded that the production of BMAA is a function of growth conditions and life cycle stages. In this study no BMAA was detected in any of the growth phases and for any of the three species tested, as either freely intracellular BMAA or protein-bound BMAA. It is possible that under laboratory

conditions cyanobacteria lose their toxicity and no longer produce secondary metabolites in detectable quantities (Schatz et al., 2005), or that this species may not produce it under most growth conditions.

In this study, no BMAA production was observed in cellular contents of *M. aeruginosa* grown under nutrient limited conditions, either as freely intracellular or protein-bound BMAA. It has been suggested in previous studies that nutrient limitation could be a prime factor in cyanobacterial BMAA synthesis (Downing et al., 2011; Monteiro et al., 2016). It has been reported that nitrogen starvation in a species of *Microcystis* yielded an increase in free cellular BMAA production (Downing et al., 2011). Moreover, the addition of $NO_3^-$ and $NH_4^+$ to starved *Microcystis* cells decreased the concentration of free cellular BMAA (Downing et al., 2011). It has been suggested that the breakdown of BMAA may provide nitrogen to starved cyanobacterial cells, and that BMAA is potentially made to promote cell survival during periods of nitrogen starvation (Downing et al., 2011).

In other studies, it has been reported that nutrient stress does not always yield BMAA production (Fan et al., 2015; Monteiro et al., 2016). Fan et al. (2015) grew two strains of *Microcystis* under varying nitrate, phosphate, light, and temperature conditions. BMAA was not detected in any species under any growth condition in this study, though the production of diaminobutyric acid (DAB) was enhanced by adverse growth conditions (Fan et al., 2015). DAB was detected in 13 of the 17 additional cyanobacteria species analyzed and in the three field species of *Microcystis*, suggesting that this isomer can be produced by cyanobacteria rather than BMAA (Fan et al., 2015). Monteiro et al. (2016) also varied the conditions under which *Nostoc* sp. grew in the laboratory, altering the salinity of the media as well as its nitrogen supply. *Nostoc* sp. did produce BMAA (between approximately 2-5 μg/g) in marine

and freshwater media, both with and without nitrogen supply. However, out of 23 strains of cyanobacteria grown in this study, BMAA was only detected in *Myxosarcina* sp. and *Nostoc* sp. (Monteiro et al., 2016). Additionally, *Nostoc* sp. produced greater concentrations of BMAA in the presence of nitrogen, as opposed to when cultured without it (Monteiro et al., 2016). Therefore, it cannot confidently be concluded that nutrient depletion promotes the production of BMAA, and the reasons for its variable production under optimal conditions in the laboratory remain unknown.

Similar studies have focused on altering growth conditions by monitoring the growth and toxin production of several toxin-producing *Anabaena* strains under light limited conditions and varying temperatures (Rapala and Sivonen, 1998). It was found that the neurotoxin, anatoxin-a, was produced most effectively when the species was grown under suboptimal light and temperature conditions (Rapala and Sivonen, 1998). However, microcystins appeared under the highest light conditions at all temperatures (Rapala and Sivonen, 1998). Similarly, Hobson and Fallowfield (2003) found that toxin production and intracellular toxin concentrations were highest when *Nodularia spumigena* was grown in low light conditions and at salinities and temperatures greater than or equal to the location of isolation, Lake Alexandria, Australia. It is therefore possible that light, temperature, and salinity control whether the bloom becomes hepatotoxic or neurotoxic in nature, and overall toxin production, and these factors should be considered for future analysis of cyanobacteria that produce different toxin types, particularly BMAA, as they were not included in the present study (Rapala and Sivonen, 1998).

Although BMAA was not observed in either free or cellularly bound forms, the presence of contaminating bacteria in all three cultures raises the possibility that they might

impact dissolved concentrations. It is known that many species of marine bacteria often degrade peptide chains which are assimilated by the bacteria, or degraded further into the amino acid components, for maintenance and cellular function (Harvey et al., 2006; Liu et al., 2017). Bacterial decomposition of amino acid peptides aids in the carbon and nitrogen cycles in the ocean, and helps to regenerate nutrients (Liu et al., 2017). Bacteria also consume amino acids in aquatic environments to acquire nutrients and carbon for growth (Kirchman, 1994). Alphaproteobacteria are often the most common phyla in ocean environments, and *Cytophaga*-like bacteria inhabit freshwater and marine environments (Harvey et al., 2006). Several genera of bacteria belonging to the Proteobacteria phylum are known to be capable of degrading cyanobacterial toxins, including *Methylobacillus, Paucibacter, Arthrobacter, Bacillus,* and *Lactobacillus* (Kormas and Lymperopoulou, 2013). Bacterial species belonging to the genera *Sphingomonas, Sphingosinicella, Arthrobacter, Brevibacterium, Rhodococcus,* and *Burkholderia* are capable of degrading microcystins and nodularins within several days (Kormas and Lymperopoulou, 2013). There is little information reported regarding the degradation of neurotoxins, though it has been reported that anatoxins can be degraded by *Pseudomonas* sp. (Kormas and Lymperopoulou, 2013). As a simple organic molecule, BMAA has the potential to be consumed or degraded by marine bacterial species (Kormas and Lymperopoulou, 2013). I did not detect any BMAA produced by any cyanobacterial species in either the freely intracellular form or the protein-bound form, and experimental cultures were not axenic (Table 5). It is therefore possible that the bacteria present were removing BMAA that may have been produced and released by the cyanobacteria before detection, although this does not explain the absence of BMAA bound as cellular protein.

*Methodological considerations*

There has been substantial debate over the most appropriate analytical techniques for detecting BMAA in cyanobacterial cells and tissue samples. There appears to be broad agreement that the use of HPLC-MS/MS is the most effective approach to identify BMAA and related compounds (Jonasson et al., 2010; Spácil et al., 2010). This is because it is sensitive, unequivocally identifies BMAA in complex biological matrices, and allows the separation of BMAA from structurally similar isomers and compounds that is not possible with other methods (Jonasson et al., 2010; Spácil et al., 2010). Earlier studies using a gradient HPLC system and fluorescence detection to determine the concentration of BMAA and other structurally similar compounds (Cox et al., 2003 and 2005) have been criticized as they are unable to analytically distinguish between BMAA and its isomer DAB (Jonasson et al., 2010). An earlier study reporting that nearly all cyanobacteria species are capable of producing BMAA (Cox et al. 2005) may therefore have over-reported BMAA production (Jonasson et al., 2010). This suggests that the BMAA detection in some earlier studies (e.g., Cox et al. 2003, 2005) may have overestimated BMAA production due to the coelution of other similar compounds and the positive results from these studies may have been due to the application of methods that could not unequivocally identify BMAA or analytically separate it from structurally similar compounds.

A more recent work by Jonasson et al. (2010) employed HPLC-MS/MS to distinguish BMAA and DAB produced by cyanobacteria (Jonasson et al. 2010). These investigators performed a solid phase extraction of BMAA and precolumn derivatization with AccQ-Tag before subsequent analysis by HPLC-MS/MS. This method allowed for the identification of BMAA from a range of biological matrices with a very low limit of detection of 70 fmol, or

8.26 pg, effectively eliminating the potential for a false-positive reading. Using this method, Jonasson et al. (2010) detected 0.001 to 0.015 μg BMAA/g dry weight in cyanobacteria and 6-fold higher concentrations in zooplankton, and between 0.99 to 1.29 μg BMAA/g dry weight in the brain tissue of fish species *S. maximus*, concentrations much lower than those reported by Cox et al. (2005) (Table 2). The methods used by Jonasson et al. (2010) are very similar to the ones used in the present study, with one exception. Filippino et al. (unpublished data, personal communication) were able to avoid the derivatization step by using the ion pairing reagent HFBA, the rapid separation liquid chromatography (RSLC) column, and the Xcalibur mass selection software to separate and isolate individual amino acid peaks without the use of derivatization, and this may increase the sensitivity of the instrument and reduce the possibility of misidentification of BMAA. The present study utilized the same methodology as the Filippino et al. (unpublished data, personal communication) study to increase instrument sensitivity without the use of derivatization.

Due to recurring discrepancies regarding which method of detection should be used, Spácil et al. (2010) proposed a standard method. They stated that a BMAA analysis needs to include a cell lysis and acid hydrolysis step to break down proteins, a solid phase extraction step using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), and liquid chromatography followed by tandem MS/MS detection of chemical constituents (Spácil et al., 2010). This method allows for the simultaneous identification of BMAA and DAB and the detection of these compounds in complex biological samples. Because the methodology used in this study employs the best practices outlined by Jonasson et al. (2010) and Spácil et al. (2010), the absence of BMAA across the species and culture conditions examined here suggest that these organisms may not produce BMAA, as established previously (Cox et al. 2005).

# CHAPTER III
# THE POTENTIAL FOR BIOACCUMULATION OF BMAA IN BLUE CRAB TISSUES

## Introduction

### *Cyanobacterial toxins in marine organisms*

Cyanobacterial blooms often occur in Chesapeake Bay tributaries, with toxic blooms appearing in the summer months (Gilbert et al. 2001; Klemas, 2012). Microcystin producing blooms have been frequently encountered in freshwater rivers like the Bush, Sassafras, Potomac, and Transquaking rivers (Tango and Butler, 2008; Preece et al., 2017). Saxitoxins produced by the cyanobacteria *Aphanizomenon flos-aquae* have also been detected in the Sassafras River (Tango and Butler, 2008). Other toxin-producing cyanobacteria commonly found in the tributaries of the Chesapeake Bay include the genera *Anabaena, Aphanizomenon flos-aquae, Microcystis,* and *Cylindrospermopsis* (Tango and Butler, 2008).

Toxins produced by cyanobacteria can yield detrimental effects on the health and behavior of aquatic organisms by causing higher death rates, inhibiting feeding, and promoting paralysis, as well as reducing their growth and fecundity (Ferrão-Filho and Kozlowsky-Suzuki, 2011). Hepatotoxin accumulation has been reported in organisms of all trophic levels, beginning with primary consumers such as zooplankton (Ferrão-Filho and Kozlowsky-Suzuki, 2011). A study concentrating on microcystin accumulation in organisms inhabiting four Swedish lakes reported microcystin concentrations in zooplankton as high as 9 µg/g, in benthic crustaceans and bivalves at a concentration of 10 µg/g, and smelt liver at a concentration of 2.7 µg/g (Larson et al., 2017). This study concluded that although microcystins are transferred throughout the food web, they are highly concentrated in zooplankton and zoo-benthic organisms (Larson et al., 2017). Additionally, a study by Kim et

al. (2017) showed that three species of filter feeding bivalves, *Sinanodonta woodiana*, *Sinanodonta arcaeformis*, and *Unio douglasiae*, that consume cyanobacteria had microcystins in their tissues.

It is uncommon for dermatoxins like aplysiatoxins and lyngbyatoxin-a to transfer in an aquatic food web, as aquatic organisms often have a protective outer layer preventing dermal toxins from entering their bodies (Sivonen, 2009; Ferrão-Filho and Kozlowsky-Suzuki, 2011). Besides hepatotoxins, neurotoxins are the other main cyanotoxin that is passed through an aquatic food web by means of ingestion (Ferrão-Filho and Kozlowsky-Suzuki, 2011). Like hepatotoxins, certain neurotoxins can accumulate in the tissues of cyanobacteria consumers and be passed to higher trophic levels (Ferrão-Filho and Kozlowsky-Suzuki, 2011). Haney et al. (1995) observed reduced movement in the thoracic appendages and an increased rejection rate of consumed particles by the post-abdomen of the zooplankton species *Daphnia carinata* after exposure to saxitoxins. Bivalves have also been examined as a vector for transmitting saxitoxins, more specifically, PSP, to crabs after they had been fed toxic strains of *Alexandrium* (Jester et al., 2009). It was determined that paddle crabs that were fed the PSP affected bivalves had accumulated the toxin themselves in their visceral tissues, which includes the digestive system, hepatopancreas, and gonads (Jester et al., 2009).

### *Cyanotoxins in blue crabs*

Due to their varied diet, blue crabs may ingest organisms that have bioaccumulated cyanobacterial toxins, including BMAA, into their tissues (Brand, et al., 2010; Belgrad and Griffen, 2016). There are several organs in the blue crab that are important to analyze for contaminants, like BMAA, as they may contain a higher concentration of toxins than others.

It is first important to examine the presence of any toxins in the blue crabs stomach to determine if they have ingested any toxins, either by directly or indirectly feeding on contaminated organisms, as survival is controlled by diet (Brand, et al., 2010; Belgrad and Griffen, 2016).

Another organ that is highly capable of accumulating toxins is the hepatopancreas, as this organ is responsible for absorbing and storing nutrients, in addition to digestion (Wang et al., 2014). The concentration of microcystins has been examined in blue crab tissues, and when compared to muscle and viscera tissues, the hepatopancreas had the highest concentration of 820 µg/kg wet weight tissue (Garcia et al., 2010). Neurotoxins can also accumulate, as Torgersen et al. (2005) examined the concentrations of the diarrhetic shellfish poisoning (DSP) neurotoxin in various brown crab tissues and found the highest concentrations in the hepatopancreas.

Several species of crabs, including *Cancer productus, Pugettia producta* and *Zosimus aeneus*, have tested positive for saxitoxins in their muscle tissues during dinoflagellate blooms (Deeds et al., 2008). Therefore, there is the potential for other neurotoxins, like BMAA, to accumulate in blue crab meat, and potentially enter the bodies of human consumers.

*BMAA accumulation in blue crab tissues*

Like other cyanotoxins, BMAA is capable of being passed to higher trophic levels by ingestion of contaminated prey (Salomonsson et al., 2015). Specifically, BMAA can be passed to blue crabs either by direct ingestion of toxic algae, or by their main prey, bivalves, as they efficiently filter toxic cyanobacteria from the water (Ferrão-Filho and Kozlowsky-

Suzuki, 2011; Belgrad and Griffen, 2016). Several previous studies have reported BMAA concentrations ranging from 0.056 to 2286 µg/g in different aquatic organisms, using either HPLC system and fluorescence detection (LC/FD) or HPLC-MS/MS (LC/MS/MS) techniques (Table 6) (Christensen et al., 2012). Salomonsson et al. (2015) reported BMAA concentrations ranging from 0.29-7.08 µg/g wet weight tissue in mussels from a Swedish fish market. As these bivalves filtered the water for nourishment, they also filtered and consumed cyanobacteria, subsequently incorporating cyanobacterial BMAA into their tissues (Salomonsson et al., 2015). Bivalves also filter and consume heterotrophic nanoflagellates and ciliates, direct predators of phytoplankton, thus ingesting the contaminated zooplankton that may have incorporated BMAA into their own cells (Masseret et al., 2013). This presents a trophic pathway for the contamination of higher trophic level organisms, like blue crabs, who prey upon the BMAA contaminated tissues of bivalves (Salomonsson et al., 2015).

**Table 6.** Concentrations of BMAA detected in invertebrates and the methodology used. (Table is adapted from Christensen et al., 2012 (and references therein), Table 3 "Comparison of BMAA concentrations determined for various aquatic invertebrates (References: 1 = Brand et al., 2010; 2 =Jonasson et al., 2010; and 3 = this study)".

| **Species (common name)** | **Sample Location** | **Mean BMAA (µg/g)** | **Method** | **Reference** |
|---|---|---|---|---|
| *Callinectes sapidus* (blue crab) | South Florida | 2286 | LC/FD | 1 |
| *Callinectes sapidus* (blue crab) | Biscayne Bay, Florida | 2275 | LC/FD | 1 |
| *Pinctada margaritifera* (oyster) | South Florida | 275 | LC/FD | 1 |
| *Crassostra virginica* (oyster) | Caloosahatchee River | 293 | LC/FD | 1 |
| *Utterbackia imbecillis* (mussel) | Caloosahatchee River | 256 | LC/FD | 1 |
| *Ostrea edulis* (oyster) | Kattegat Sea | 0.056 | LC/MS/MS | 2 |
| *Mytilus edulis* (mussel) | Kattegat Sea | 0.179 | LC/MS/MS | 2 |
| *Callinectes sapidus* (blue crab) | North Florida | 8.7 | LC/MS/MS | 3 |

BMAA has been detected in various blue crab organs (Brand et al., 2010; Christensen et al., 2012; Field et al., 2013). It has been reported in blue crab hepatopancreas tissue supernatant in concentrations as high as 9.5 μg/g dry weight (Field et al., 2013). This organ not only accumulates harmful contaminants like BMAA, but acts as a conduit to other essential organs. Field et al. (2013) also reported 29.6 μg/g dry weight of BMAA detected in blue crab backfin muscle tissue supernatant, while other studies have reported BMAA ranging between 5-47 μg/g dry weight in blue crab muscle, with both results obtained using HPLC-MS/MS methodology (Christensen et al., 2012). Lastly, Brand et al. (2010) reported 6976 μg/g dry weight of BMAA detected in blue crab muscle tissues collected from Biscayne Bay, FL.

As BMAA has been reported to accumulate in blue crabs and other aquatic invertebrates, it can also be transferred to apex predators, including humans (Masseret et al., 2013). Human exposure to BMAA is likely to increase as toxic cyanobacterial blooms are projected to occur more frequently as a result of climate change (Paerl and Paul, 2012; Paerl and Otten, 2013).

Because BMAA is a free and protein-bound amino acid, it is necessary to know the protein content of tissue. The proximate composition of the blue crab claw muscle tissue protein has been reported as ranging from 14.86 to 19.55% (Farragut, 1965; Küçükgülmez et al., 2006; Zotti et al., 2016). Wu et al. (2010) estimated that the protein content of blue crab hepatopancreas tissue was between 8.4 and 10.8 + 0.8 % between male and female crabs. However, Küçükgülmez et al. (2006) estimated the hepatopancreas protein content to be approximately 18.81%.

*Blue crabs in the Chesapeake Bay*

The Chesapeake Bay is responsible for approximately half of the United States blue crab harvest annually and supports the largest blue crab fishery in North America (Prager, 1996; Jensen et al., 2005). Although blue crabs are tolerant of a wide range of temperatures and salinities, during the winter months both males and female remain in the deeper portions of the Bay and often submerge themselves in the mud (Jensen et al., 2005). As temperatures warm during the spring, male and female crabs meet in the Bay's mesohaline areas and in tributaries (Eggleston et al., 2015). Crabs mate and spawn from May to October and the female is fertilized during the terminal molt while the shell is soft (Eggleston et al., 2015). After mating, the male will remain in these regions, while the female will migrate to higher salinities at the mouth of the Bay (Eggleston et al., 2015). Once at the mouth of the Bay, the females will spawn and release their larvae, which will begin to grow and molt several times in higher salinity waters offshore (Eggleston et al., 2015).

While the females continue to migrate, males remain in lower salinity waters to continue searching for other suitable mates (Key et al., 1997). This could potentially prove disadvantageous for males as cyanobacterial blooms are more common in lower salinity waters during spring and summer months (Gilbert et al., 2001). Additionally, in the Chesapeake Bay, large male crabs are coveted by watermen since they do not migrate like females (Key et al., 1997; Abbe, 2002). Since males remain in potentially contaminated waters for their adult lives, their potential to accumulate toxins into their tissues is greater than females. Further, because restaurants in the Chesapeake Bay region most commonly provide adult males, the chance that humans consume contaminated blue crabs is higher (Abbe, 2002).

The migration of both male and female blue crabs throughout the Chesapeake Bay over the course of a year allows for exposure to a wide variety of contaminants that may exist along their migratory routes. For females, this migration may include traveling distances as great as 200 km, which is energy costly, but is necessary for them to attain safety from predators, a greater selection of mates, and favorable temperature and salinity waters for their larvae to survive (Turner et al., 2003). Conversely, migrating blue crabs, especially females, may be able to escape pulses of unfavorable conditions, like excess heavy metals in the water, or hypoxia, more easily than sessile organisms, and ensure that they are not exposed to adverse conditions for long enough periods to affect their survival (Tankersley and Wieber, 2000; Belgrad and Griffen, 2016).

*Blue crab diet and feeding patterns*

Blue crabs have a diverse diet ranging from invertebrates and sessile infauna to detritus and plant material (Belgrad and Griffen, 2016). While stomach contents suggest blue crabs prey on mollusks, arthropods, chordates, and annelids, they also forage on dead organisms, detritus, and benthic algae (Belgrad and Griffen, 2016). The blue crab diet is extremely important for their survival and reproduction as it controls their metabolism and homeostasis, and diet alteration may eventually lead to species decline (Belgrad and Griffen, 2016). However, feeding behavior does change throughout a blue crab's life cycle, and throughout different seasons during the year (Belgrad and Griffen, 2016). Blue crab diets are also heavily influenced by habitat and salinity and it has been reported that more gastropods, plant matter, and detritus are ingested at lower salinity areas and more shrimp, fish, and crabs are eaten at higher salinity areas (Laughlin, 1982). While blue crab preference to certain prey items may exist based on nutritional value, it is also possible that blue crabs can detect when

prey is contaminated or unsafe to eat (Weissburg et al., 2012). Blue crabs can detect prey based on their chemical components like amino acids, sugars, and nucleotides and they are able to distinguish between healthy prey and contaminated prey (Weissburg et al., 2012). The crabs detect aversive odors that harmful food exhibit and cease foraging when metabolites from injured prey are sensed (Weissburg et al., 2012). However, there is no information as to whether blue crabs can detect or avoid BMAA.

*Physiology of the blue crab digestive system*

In order to understand how toxic substances may accumulate in blue crab tissues, it is important to consider the path of potential bioaccumulation of BMAA by blue crabs, beginning with the ingestion of contaminated prey items. Ingested food is directly deposited into the stomach of the crab after passing through the mouth parts and esophagus (McGaw and Reiber, 2000). Another important organ is the hepatopancreas, which is responsible for enzymatic digestion of food (McGaw and Reiber, 2000). It is important to examine the blue crab muscle tissue, particularly the backfin muscle, as this tissue is most frequently consumed by humans, and so offers the greatest toxin bioaccumulation risk to humans (Millikin and Williams, 1984).

After passing through the mouth parts and esophagus, ingested food is first moved into the crab's stomach, also called the foregut, which is separated into the cardiac and pyloric chambers (McGaw and Curtis, 2013). The cardiac chamber is where food is stored and eventually digested, and the pyloric chamber is responsible for sorting digested materials and ensuring that only liquid is transferred to the hepatopancreas (McGaw and Curtis, 2013). Although the stomach is not usually consumed by humans, it is an important organ to analyze

for toxin presence, as any contaminated digesta may assimilate into more frequently consumed tissues.

The hepatopancreas is infrequently consumed except in some Asian cultures where it is considered a delicacy (Jester et al., 2009). Contaminant accumulation often occurs in the hepatopancreas because food items are passed through the stomach to the hepatopancreas where they are digested and metabolized (Belgrad and Griffen, 2016). For example, the hepatopancreas functions as the pancreas and liver in blue crabs, and aids in the secretion of enzymes, as well as the storage and absorption of nutrients (Wang et al., 2014; Cervellione et al., 2017). The nutrients obtained from food are also stored in the hepatopancreas and eventually distributed to other organs including the muscles and gonads to aid in growth and reproduction (Wang et al., 2014). Finally, it is important to consider the contamination of blue crab muscle tissue which is most often consumed by humans. The backfin muscle tissue is the blue crabs strongest and largest muscle, as it is utilized for swimming. Because potentially contaminated digesta are distributed from the hepatopancreas to the muscle in blue crabs, there is the possibility for predators, including humans, to consume this contaminated meat.

In this study, the BMAA concentrations in various blue crab tissues were examined. Specifically, concentrations of BMAA were measured in natural populations of cyanobacteria and in blue crabs potentially exposed to these populations. Concentrations of BMAA were measured in muscle, hepatopancreas, and stomach tissues of blue crabs. It was hypothesized that if cyanobacteria in the upper and lower regions of the Chesapeake Bay produce BMAA *in situ*, then BMAA would accumulate in the digestive and muscle tissues of Chesapeake Bay blue crabs.

## Methods

*Sample collections*

Mixed cyanobacteria assemblage samples from Budd's Landing, and samples of *Oscillatoria limnosa* and benthic hydrilla potentially including cyanobacterial epiphytes from Lloyd's Creek, branches of the Sassafras River, were collected by Maryland Department of Natural Resources (MD DNR) (Figure 18) (C. Wazniak- Maryland DNR).

Blue crabs were collected from 3 sites in the Chesapeake Bay. Benthic trawls at several stations within the Lower York River, a tributary of the Chesapeake Bay were performed on 7/9/18 on the *R/V Tidewater*, a Virginia Institute of Marine Science (VIMS) vessel. These crabs were collected from areas that had not been exposed to cyanobacterial blooms (Figure 19). Crabs were purchased on 9/17/18 in Berlin, MD that were collected from the Miles River, a tributary of the upper Chesapeake Bay (Figure 19). These crabs were collected during a time when extensive *Microcystis*, *Lyngbia*, and *Oscillatoria limnosa* cyanobacteria HABs and *Margalefidinium polykrikoides* dinoflagellate HABs were reported throughout the Chesapeake Bay (C. Wazniak- Maryland DNR and T. Tuckey- VIMS, personal communication). On 9/21/18, crabs were collected from the Rhode River, a northeastern tributary of Chesapeake Bay, by Robert Aguilar at the Smithsonian Environmental Research Center, Edgewater MD (Figure 19).

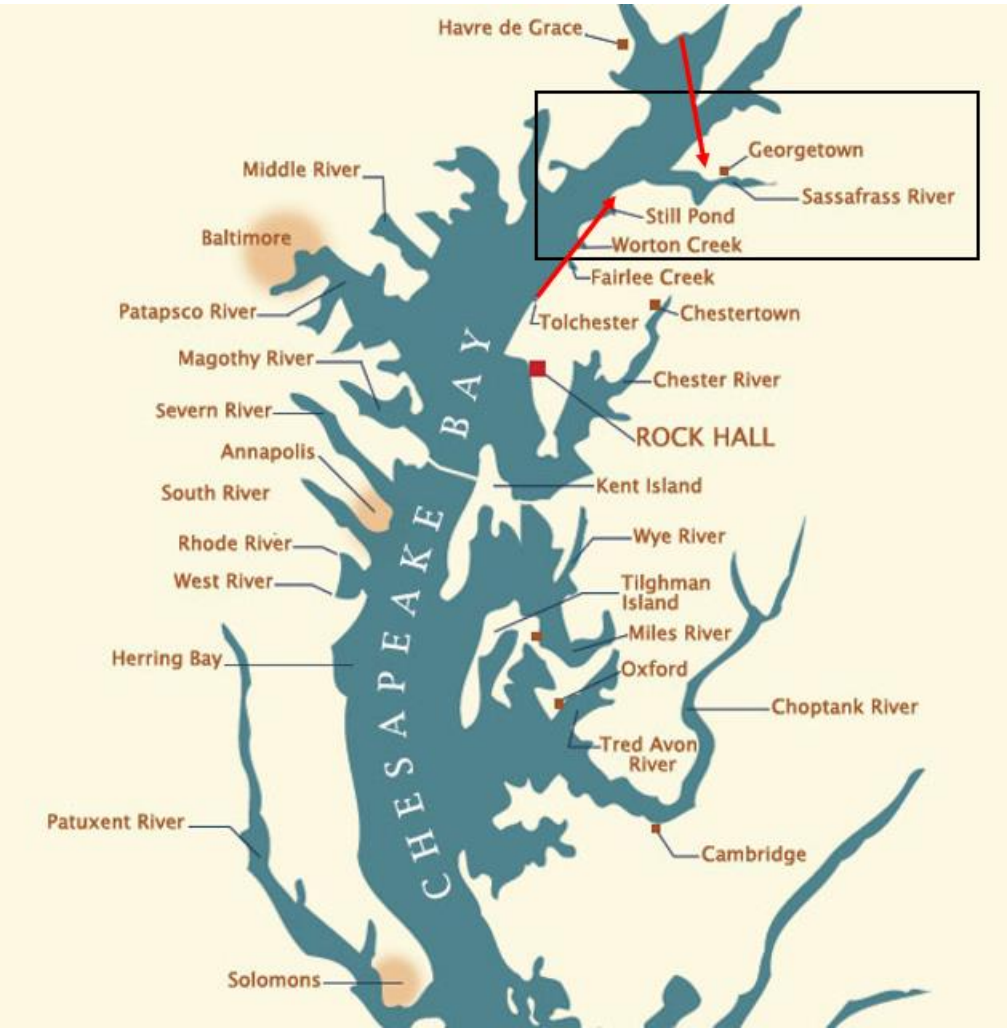


**Figure 18.** Collection sites of wild cyanobacteria samples in the Sassafras River. Mixed cyanobacteria samples were from Budd's Landing (top arrow) and *Oscillatoria limnosa* and benthic hydrilla potentially including cyanobacterial epiphytes were from Lloyd's Creek (bottom arrow).


**Figure 19.** Sites of cyanobacteria and blue crab collection in the Chesapeake Bay. The black square represents areas where cyanobacterial blooms are common during summer months (Tango and Butler, 2008; Preece et al., 2017).

Crabs were kept alive on ice until dissection began the day following collection/delivery. For each dissected crab, the following data were recorded: crab number, date of collection, collection location, sex, carapace width (mm), muscle wet weight (g), hepatopancreas wet weight (g), and stomach wet weight (g) (Table 7). The largest crabs were chosen for analysis from each sample location regardless of sex. Nineteen male and 8 female crabs were dissected in total, with an average carapace width of 143.37 $\pm$ 4.54 mm and 134 $\pm$ 8.48 mm, respectively. The average male and female muscle tissue weights were 14.1 $\pm$ 0.86 g and 7.35 $\pm$ 1.07 g, respectively. The average male and female hepatopancreas tissue weights were 7.7 $\pm$ 0.7 g and 3.9 $\pm$ 0.93 g, respectively and the average stomach tissues were 2.4 $\pm$ 0.25 g and 1.55 $\pm$ 0.24 g, respectively. Backfin muscle tissue, hepatopancreas, and the stomach and contents were removed from each crab and stored separately in plastic bags at -80 °C until analysis.

**Table 7.** Blue crab tissue samples analyzed as part of this study.

| Crab Number | Date of Collection | Collection Location | Sex | Carapace Width (mm) | Muscle (g) | Hepatopancreas (g) | Stomach (g) |
|---|---|---|---|---|---|---|---|
| 1 | 7/9/2018 | York River | Male | 102 | 7.3 | 4.1 | 2.8 |
| 2 | 7/9/2018 | York River | Female | 129 | 6.9 | 3.5 | 2 |
| 3 | 7/9/2018 | York River | Male | 113 | 10.4 | 7 | 0.8 |
| 4 | 7/9/2018 | York River | Female | 129 | 4.2 | 2.2 | 0.9 |
| 5 | 7/9/2018 | York River | Male | 114 | 9.5 | 3.6 | 0.9 |
| 6 | 7/9/2018 | York River | Male | 134 | 12.8 | 5.6 | 1.9 |
| 7 | 7/9/2018 | York River | Male | 122 | 8 | 6.3 | 2.9 |
| 8 | 7/9/2018 | York River | Male | 116 | 8.9 | 4.7 | 1.1 |
| 9 | 7/9/2018 | York River | Female | 132 | 8.1 | 3.9 | 1.8 |

Table 7 Continued.

| Crab Number | Date of Collection | Collection Location | Sex | Carapace Width (mm) | Muscle (g) | Hepatopancreas (g) | Stomach (g) |
|---|---|---|---|---|---|---|---|
| 10 | 7/9/2018 | York River | Female | 89 | 3.5 | 1.2 | 0.6 |
| 11 | 7/9/2018 | York River | Female | 125 | 7.1 | 2.2 | 1.9 |
| 12 | 7/9/2018 | York River | Female | 140 | 6.4 | 5.1 | 1.5 |
| 13 | 9/17/2018 | Miles River | Male | 150 | 13.1 | 13.3 | 2.4 |
| 14 | 9/17/2018 | Miles River | Male | 149 | 15.2 | 10.8 | 3.5 |
| 15 | 9/17/2018 | Miles River | Male | 150 | 17.1 | 5.3 | 1.6 |
| 16 | 9/17/2018 | Miles River | Male | 160 | 16.9 | 12.8 | 2.6 |
| 17 | 9/17/2018 | Miles River | Male | 156 | 17 | 12.3 | 2.9 |
| 18 | 9/17/2018 | Miles River | Male | 150 | 14.9 | 4.9 | 1.3 |
| 19 | 9/17/2018 | Miles River | Male | 163 | 17.9 | 8 | 3.8 |
| 20 | 9/17/2018 | Miles River | Male | 159 | 19.3 | 10.3 | 1.9 |
| 21 | 9/17/2018 | Miles River | Male | 152 | 16.5 | 8.2 | 4.5 |
| 22 | 9/17/2018 | Miles River | Male | 157 | 19.6 | 9.9 | 2.2 |
| 23 | 9/21/2018 | Rhode River | Male | 165 | 13.3 | 4.4 | 2.7 |
| 24 | 9/21/2018 | Rhode River | Male | 154 | 15.2 | 7.7 | 1.6 |
| 25 | 9/21/2018 | Rhode River | Female | 160 | 13.1 | 9.7 | 2.7 |
| 26 | 9/21/2018 | Rhode River | Male | 158 | 14.6 | 7.2 | 4.2 |
| 27 | 9/21/2018 | Rhode River | Female | 168 | 9.5 | 3.4 | 1 |

*Field cyanobacteria and animal tissue analysis*

In preparation for acid hydrolysis, cyanobacteria pellets were obtained, and each crab tissue sample was weighed, and pellets and tissues were placed in 20 mL scintillation vials, where they were homogenized with 1-2 mL of deionized water using a Teflon Homogenizer until they became a smooth paste. Approximately 5 g of muscle tissue, 2-3 g of hepatopancreas, and 0.5-2 g of stomach tissue were weighed and then placed into 4 mL amber vials and 500-1000 μL of 6 M HCl was added. The vials were immediately flushed with $N_2$ gas and sealed off with Teflon tape and placed in a 110 °C heating block for 20 hours.

After hydrolysis, approximately 500 μL of the hydrolysate was pipetted into 2 mL amber vials and dried to remove residual HCl. After drying, 20 μL of water was added and evaporated to ensure all acid was gone. This step was then repeated a second time. The samples were then stored at -80 °C prior to analysis.

On the day of analysis, 50 μL of the sample diluted to a concentration of approximately 1520 ng/mL hydrolyzed protein was re-dissolved in 100 μL of the ion-pairing reagent HFBA and 50 μL of 300 ng/mL L-Aspartic acid. All four mobile phase channels were purged to remove bubbles from binary pump and pump lines, and the Orbi-trap mass spectrometer was started 30 minutes prior to analysis to allow for equilibration.

The same method of liquid chromatography-tandem electron spray ionization mass spectrometry and mass selection software described in Chapter II was used to analyze field cyanobacterial samples and blue crab tissues. Amino acid concentrations were calibrated using an internal standard/external standard paired set to provide coverage over the major functional groups of the amino acids being determined. The only change in the calibration and

standards from Chapter II was that an individual calibration curve was constructed for BMAA and DAB and was normalized to the internal standard L-Aspartic-2,3,3-$d_3$ Acid.

*BMAA minimum detection limits for tissues*

The calibration curve ranged from the lowest limit of detection of 25 pg to 10,000 pg, and this curve and the calibration curve including both BMAA and DAB can be found in the appendix section (Figures 2-A and 3-A). The minimum detection limits for blue crab tissue samples were calculated by utilizing the instrumental limit of detection of 25 pg and the actual percentage of hydrolyzed sample that was analyzed by the instrumentation following injection. It was determined that in order to detect any BMAA in muscle tissues, there would have had to have been a concentration greater than 0.0001 µg/g wet weight tissue. For the hepatopancreas, there would have had to have been a concentration greater than 0.0002 µg/g wet weight tissue and for stomach tissue, a concentration greater than 0.0005 µg/g wet weight tissue.

*Blue crab tissue protein analysis*

For the crab tissue protein analysis, samples were prepared according to the instructions for the Thermo Scientific Pierce™ BCA (bicinchoninic acid) Protein Assay Kit. A sodium bicarbonate diluent solution with a concentration of 50 mmol was created for use in standard preparation and tissue dissolution. A set of 8 diluted bovine serum albumin (BSA) standards were prepared, with BSA protein concentrations ranging from 25 to 2000 µg/mL (Figure 4-A found in the appendix section). Using the information that muscle tissue is composed of approximately 12% protein, 80 mg of tissue was added to 20 mL of sodium bicarbonate solution to produce a protein concentration of 600 µg/mL (Farragut, 1965). Using

the estimation from Küçükgülmez et al. (2006) that the hepatopancreas is about 18% protein, 80 mg of tissue was added to 24 mL of diluent to yield a protein concentration of 600 μg/mL. Lastly, assuming that stomach tissue is low in protein, 80 mg of stomach tissue was added to 12 mL of diluent to yield a 600 μg/mL protein concentration.

The tissue solutions were then sonicated for 30 seconds and centrifuged for 20 minutes, and this process was repeated 3 times to deteriorate the protein structures within the tissues. The tissue solutions were combined with a BCA working reagent solution to prepare for analysis with a spectrophotometer. The samples were analyzed using a spectrophotometer set to a wavelength of 562 nm.

**Results**

No BMAA was detected in any of the field samples of cyanobacteria or the blue crab muscle, hepatopancreas, and stomach tissues from the three locations. Protein content of each type of tissue sampled determined that muscle tissue had an average protein content of 10.94 $\pm$ 0.63 %, hepatopancreas had an average of 9.35 $\pm$ 0.49 %, and stomach tissue had an average of 3.4 $\pm$ 0.26 % (Figure 21).

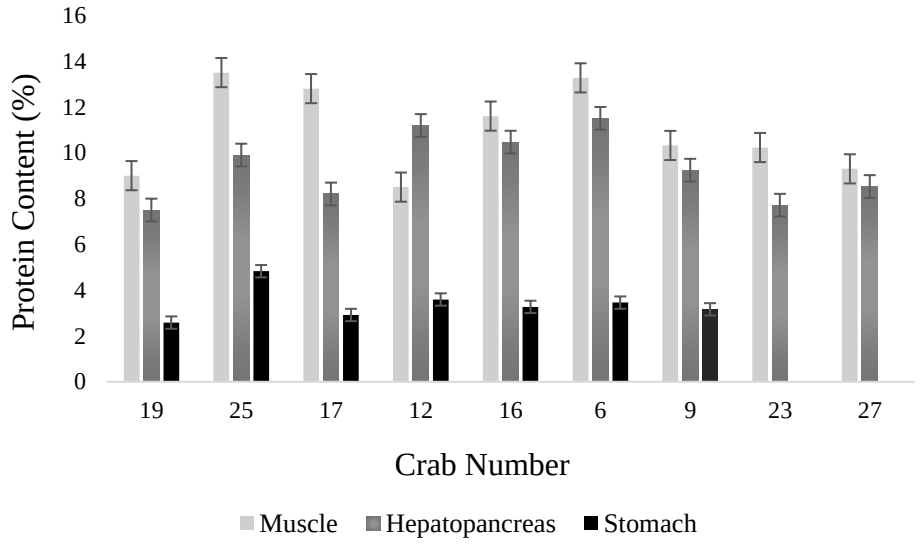


**Figure 20.** Percentage of protein in blue crab muscle, hepatopancreas, and stomach tissues, representing each collection site within the Bay.

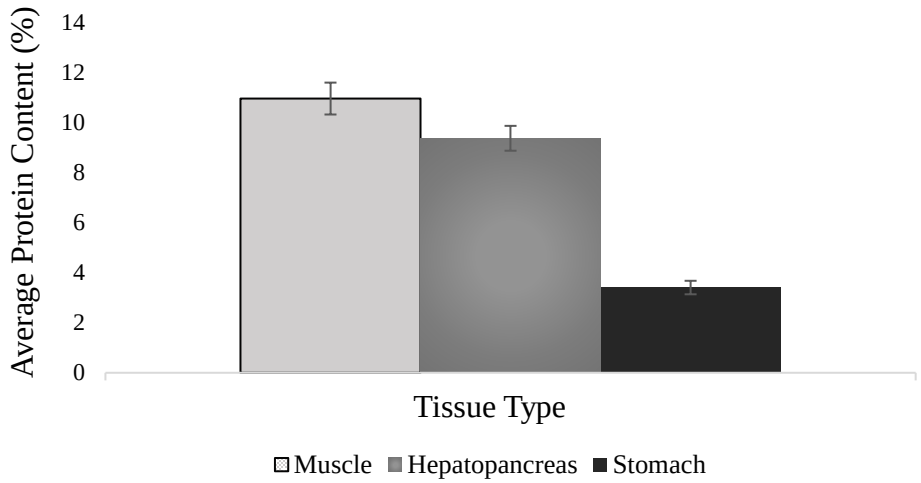


**Figure 21.** Average protein percentage of tissue types analyzed from blue crabs. Error bars represent the standard error within samples from each tissue type.

## Discussion

BMAA was not detected in any of the cyanobacterial or blue crab samples analyzed during this study. One explanation for the lack of detection of BMAA in any blue crab sample is that they did not consume any contaminated tissue or toxic cyanobacteria in the Chesapeake Bay.

Despite the detection of BMAA in Chesapeake Bay blue crabs by several previous studies, it is possible that these were isolated or sporadic events and that cyanobacteria do not produce BMAA constitutively. While Field et al. (2013) detected BMAA in 2 out of 3 blue crab claws analyzed and in the backfin muscle tissue and the hepatopancreas, these crabs were obtained from a fish market in Annapolis, MD, and their origin is unknown (Field et al., 2013). This is the only study reporting BMAA detection in blue crabs from the Chesapeake Bay and only 3 crabs were analyzed, so this study may be an exceptional case.

A second reason that no BMAA was measured in blue crab tissue is that blue crabs can identify desirable prey items using sensitive chemoreceptors that detect chemical cues released from prey in the form of amino acids, sugars, and nucleotides, among other compounds (Aggio et al., 2012; Weissburg et al., 2012). They can distinguish between attractive and aversive metabolites when they are released by prey (Weissburg et al., 2012). For example, blue crabs reduced their foraging behavior and sacrificed a feeding opportunity when in the presence of a dead conspecific, as dead crabs release injury alarm chemicals (Ferner et al., 2005). Similarly, blue crab foraging behavior was inhibited by the presence of aplysioviolin, a chemical feeding deterrent released by sea hares (Aggio et al., 2012). Avoiding lethal predation typically outweighs an opportunity to feed (Ferner et al., 2005), and though there are no current studies supporting the idea, it is possible that blue crabs are deterred by prey that have accumulated BMAA into their tissues. This is especially relevant to the present study, as no BMAA was

detected even in the stomach contents of blue crabs tested. This suggests that they are not even ingesting contaminated organisms, and therefore accumulation in other tissues is not possible.

A third possibility for the absence of BMAA in blue crabs may be that commercial fishing pressure limits accumulation (Abbe, 2002). Male blue crabs may be captured by commercial fishermen once they are of a certain carapace width in low salinity waters of the Chesapeake Bay where they aggregate to mate with females (Key et al., 1997). Mating occurs at maturity, between 12 and 18 months of age, though by this time, if they have grown large enough, they are in danger of being captured as hard-shell crabs, peeler (actively molting) crabs, and soft-shell crabs, as all stages of a crab's molt are desired by watermen, predominantly in Maryland waters, where females cannot be harvested (Paolisso, 2002). However, females residing in Virginia waters can be captured after sexual maturity has been reached. It is true that males reside in low salinity waters capable of supporting HAB growth, and the chances of them being exposed to cyanobacterial toxins and accumulating them into their tissues is greater than females (Key et al., 1997; Gilbert et al., 2001). However, capturing male blue crabs of appropriate carapace width in Maryland waters and females post-molt in Virginia waters of the Chesapeake Bay may not allow them enough exposure to BMAA before it is incorporated into their tissues, despite the chance of exposure in their habitats, as the average age of blue crabs in the Chesapeake Bay is less than 2 years (Puckett et al., 2008).

If there were to be ingestion of toxic organisms and if blue crabs were exposed to BMAA long enough for accumulation in the body to occur, there are still several other factors that may explain why BMAA was not detected in any crab tissue. It is first important to consider the conditions under which blue crab prey would be accumulating BMAA, where these organisms reside, and when they are accumulating BMAA. The diet of the blue crab mainly consists of

molluscs, in addition to arthropods and fish, and it has been reported that these organisms can accumulate BMAA into their tissues (Jonasson et al., 2010; Réveillon et al., 2015; Belgrad and Griffen, 2016). If blue crab prey were to accumulate BMAA, it would occur when cyanobacterial abundance is highest, which is typically during warmer summer months following a spring nutrient loading event (Heisler et al., 2008). However, there are no data demonstrating how long invertebrates must be exposed to cyanobacteria to incorporate BMAA into their tissues, though it is thought that constant and long exposure is needed among various organisms for the toxin to accumulate (Jiang et al., 2014a; Delcourt et al., 2018).

Microcystin exposure to organisms often yields detrimental effects soon after ingestion (Xie et al., 2005). Microcystins were detected in the bile of wild trout as soon as 1 hour after oral administration and in an additional experiment, had died within 96 hours of consuming *Microcystis* (Xie et al., 2005). *Daphnia galeata* that were fed microcystins suffered loss of appendage movement and neuromuscular communication 5-9 hours after feeding (Zanchett and Oliveira-Filho, 2013). This suggests that microcystins are fast-acting toxins that produce harmful affects to organisms within days, or even hours, of ingestion (Xie et al., 2005). It is unknown how long BMAA is retained in tissue after consumption.

Saxitoxins fed to the larvae of the crab *C. oregonensis* caused a decrease in oxygen consumption 6 hours later (Vasconcelos et al., 2010). In another study, it was reported that a dog that consumed a PSP-contaminated crab in Norfolk, England began vomiting 30 minutes post-ingestion, experienced paralysis at 40 minutes, and died at 60 minutes (Turner et al., 2018). In humans, death can occur between 2-12 hours after consumption of PSP-contaminated bivalves (Visciano et al., 2016). While there are few studies highlighting the length of exposure required for the accumulation of hepatotoxins and neurotoxins in marine organisms, the studies

referenced above suggest the effects of ingestion are severe and that these toxins are fast-acting. It is not known whether BMAA ingestion produces immediate adverse effects when ingested, though the need for chronic accumulation of misfolded proteins to produce neurological effects suggests that it does not harm organisms in the same way as other cyanobacterial toxins.

While the factors mentioned above might regulate the accumulation of BMAA in blue crab tissues, it is important to also consider blue crab metabolism. Blue crabs have an active detoxification system and are often able to expel contaminants from their bodies before accumulation in tissues (Brouwer and Lee, 2006). Therefore, it is possible that blue crabs could ingest BMAA-contaminated prey without ever incorporating it into their own tissues. While the hepatopancreas harbors BMAA, the crab's hemolymph, a fluid in the circulatory system, transports contaminants throughout the body, and may cause potential chemical accumulation in muscle tissues (Brouwer and Lee, 2006; Fredrick and Ravichandran, 2012).

After entering the crab, contaminants such as polycyclic aromatic hydrocarbons (PAHs), organohalogens, pesticides, and heavy metals either accumulate in tissues, undergo biotransformation, or are expelled from the body (Brouwer and Lee, 2006). PAHs, organohalogens, organometallics, and certain pesticides affect blue crab growth, reproduction, and development (Brouwer and Lee, 2006). The metabolism of foreign chemicals is done primarily by the hepatopancreas, which contains the enzyme system cytochrome P-450, capable of oxidizing them (Brouwer and Lee, 2006). Another exogenous chemical that is often harmful to the blue crab is the organometallic compound tributyltin (TBT), and is toxic to marine organisms as it inhibits growth (Brouwer and Lee, 2006). However, TBT is rapidly oxidized and eliminated by the blue crabs P-450 system in the hepatopancreas (Brouwer and Lee, 2006).

Cytochrome P-450 enzymes are produced in response to contaminant exposure and are responsible for detoxifying exogenous chemicals in a wide variety of organisms, ranging from prokaryotes to vertebrates (Snyder, 2000). While these enzymes also metabolize endogenous compounds like steroids and fatty acids, they are commonly used to metabolize harmful chemicals, particularly by marine invertebrates, who are exposed to toxic substances that enter marine environments (Snyder, 2000). The cytochrome P-450 enzyme system is very highly active in the blue crab stomach and hepatopancreas, which could explain why prey items contaminated with toxins, like BMAA, are never incorporated into other tissues (Brouwer and Lee, 2006).

Additionally, cytochrome P-450 enzymes can perform biotransformation of exogenous compounds and therefore detoxify the organism (Rewitz et al., 2006). When exposed to pollutants such as PAHs, cytochrome P-450 activity and production increases (Lee, 1989; Rewitz et al., 2006). PAHs occur in coastal environments as the result of crude oil production, the use of petroleum hydrocarbons, and shipping (Brouwer and Lee, 2006). When PAHs are incorporated into blue crabs by the uptake of contaminated food and water, the cytochrome P-450 system oxidizes them, conjugates them with glutathione, glucose, or sulfate, and eliminates them from the animal (Brouwer and Lee, 2006). These mechanisms allow blue crabs to survive exposure to PAHs, and could potentially aid in the elimination of BMAA as well (Rewitz et al., 2006).

As stated in Chapter II, the use of HPLC-MS/MS is the most effective approach to identify BMAA and related compounds, especially in animal tissues (Jonasson et al., 2010; Spácil et al., 2010). This is because it identifies BMAA in complex biological matrices, and distinguishes BMAA from structurally similar isomers and compounds, which is not always possible with other methods (Jonasson et al., 2010; Spácil et al., 2010). Studies that have used a

gradient HPLC system and fluorescence detection to determine the concentration of BMAA and other structurally similar compounds for aquatic animal tissues (Brand et al., 2010) may be unable to analytically distinguish between BMAA and its isomer DAB (Jonasson et al., 2010). Table 6 allows for the clear distinction between BMAA concentrations detected using the HPLC system and fluorescence detection and the HPLC-MS/MS method. Concentrations obtained in blue crab, oyster, and mussel tissues using the HPLC and fluorescence detection method from the Brand et al. (2010) study are between 2 and 5 orders of magnitude higher than the concentrations obtained in similar organisms using the HPLC-MS/MS method in the Jonasson et al. (2010) and Christensen et al. (2012) studies (Table 6). It can confidently be stated that the high concentrations reported from the Brand et al. (2010) study are a result of the inability of their methodology to properly quantify BMAA concentrations and analytically distinguish it from structurally similar compounds.

From this limited study, there is no evidence that BMAA is produced by cyanobacteria in the Chesapeake Bay or that it is bioaccumulated in Chesapeake Bay blue crabs. It is possible that BMAA is not accumulating in the tissues of blue crabs because they are not being exposed to cyanobacteria producing BMAA or due to their active metabolism and the use of the cytochrome P-450 enzymes to metabolize harmful exogenous chemicals, and eliminate these chemicals from their bodies (Brouwer and Lee, 2006).

# CHAPTER IV
# SUMMARY AND CONCLUSIONS

Cyanobacterial blooms occur regularly in Chesapeake Bay tributaries, and those that produce toxins appear to be increasing due to a warming climate, increasing nutrient loading, and runoff (Gilbert et al. 2001; Brand et al., 2010; Klemas, 2012; Paerl and Paul, 2012; Carmichael, 2013). It has been previously reported that nearly all species of cyanobacteria produce BMAA, and there has also been report of the biomagnification of BMAA in the food chain (Cox et al., 2003; Cox et al., 2005). The reasons for BMAA production remain unknown, though it has been speculated that its production is a function of growth conditions and life cycle stages (Cox et al., 2005; Esterhuizen and Downing, 2008). This present study examined the growth of three cyanobacterial species —*Microcystis aeruginosa*, *Synechococcus bacillaris*, and *Nostoc sp.* — grown under nutrient replete conditions, and *Microcystis aeruginosa* grown under nitrogen and phosphorus deplete conditions.

Across all cyanobacterial culture samples it was determined that BMAA was not present during multiple growth phases across all three species tested in either freely intracellular or protein-bound fractions. Further, no BMAA was detected in samples collected from natural populations. One explanation for this result is that the species involved in this study did not produce BMAA or that laboratory conditions were not conducive to BMAA production. The fact that nutrient depletion did not result in the production of BMAA contrasts with that from one previous study (Downing et al., 2011), suggesting additional work is needed.

It has been reported that toxin production and intracellular toxin concentrations were highest when cyanobacterial species *Nodularia spumigena* was grown in low light conditions and at salinities and temperatures greater than or equal to the location of isolation, Lake

Alexandria, Australia (Hobson and Fallowfield, 2003). It is therefore possible that light, temperature, and salinity control overall toxin production and whether the bloom becomes hepatotoxic or neurotoxic in nature. These factors should be considered for future analysis of cyanobacteria that produce different toxin types, particularly BMAA, as they were not included in the present study (Rapala and Sivonen, 1998).

Early studies suggesting that BMAA production is nearly ubiquitous among cyanobacteria were conducted using analytical methods that may not distinguish between compounds with similar structures. There is growing agreement that the use of HPLC-MS/MS is the most effective method to unequivocally separate and quantify BMAA and related compounds (Jonasson et al., 2010; Spácil et al., 2010) in samples from many matrices. We have improved on these methods by eliminating the derivatization step which may increase the sensitivity of HPLC-MS/MS to detect BMAA and reduce the possibility of misidentification (Filippino et al., unpublished data). The use of HPLC-MS/MS methods with or without the use of derivatization is recommended to prevent the misidentification of BMAA and overestimation of this compound.

One explanation for the lack of BMAA in any blue crab tissue is that they did not consume any contaminated prey or toxic cyanobacteria. This may be because cyanobacteria are not producing BMAA or that blue crabs can identify desirable prey items using sensitive chemoreceptors that detect chemical cues released from prey in the form of amino acids, sugars, and nucleotides. Those containing BMAA may be deemed unfit for consumption (Aggio et al., 2012; Weissburg et al., 2012). Another reason blue crabs may not be incorporating BMAA into their tissues in the Chesapeake Bay is because commercial fishing pressure is so great that they are being collected before adequate exposure occurs, thus preventing accumulation (Abbe, 2002).

Blue crabs have an active detoxification system and are often able to metabolize and reject contaminants from their bodies before accumulation can occur (Brouwer and Lee, 2006). Therefore, it is possible that blue crabs could ingest BMAA-contaminated prey without ever incorporating it into their own tissues. The metabolism of foreign chemicals is done primarily by the hepatopancreas, which contains the enzyme system cytochrome P-450, capable of oxidizing contaminants (Brouwer and Lee, 2006). Cytochrome P-450 enzymes are produced in response to contaminant exposure and are responsible for detoxifying exogenous chemicals in a wide variety of organisms, which could potentially occur to BMAA after ingestion by blue crabs (Snyder, 2000).

BMAA was not detected in any cyanobacteria species grown under varying laboratory conditions, wild cyanobacteria sample, or blue crab tissue analyzed in this study. Though these results suggest that BMAA is not present in the Chesapeake Bay food web, it cannot be proven that BMAA is not a factor in the trophic ecology of this estuary. Further analysis is required to concretely define the presence of BMAA in the Chesapeake Bay ecosystem.

Future analysis might test the response of animals to BMAA ingestion through direct exposure of blue crabs to BMAA in food items to determine the length of exposure required for BMAA accumulation in tissues. This analysis should also focus on the accumulation of BMAA in blue crab prey and the length of exposure required to detect BMAA in those organisms as well. Future studies should try to target areas in the Chesapeake Bay where blue crabs and their prey are being exposed to BMAA, and seasons in which cyanobacteria are most abundant, e.g., summer. Controlled feeding experiments with BMAA-spiked food pellets could also elucidate whether food items containing BMAA ever pass from the stomach to the hepatopancreas, to

accumulate in other tissues (e.g., muscle). Finally, HPLC-MS/MS methods, with or without the use of derivatization, should always be used to accurately identify and quantify BMAA concentrations.

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# APPENDICES

## CHAPTER II

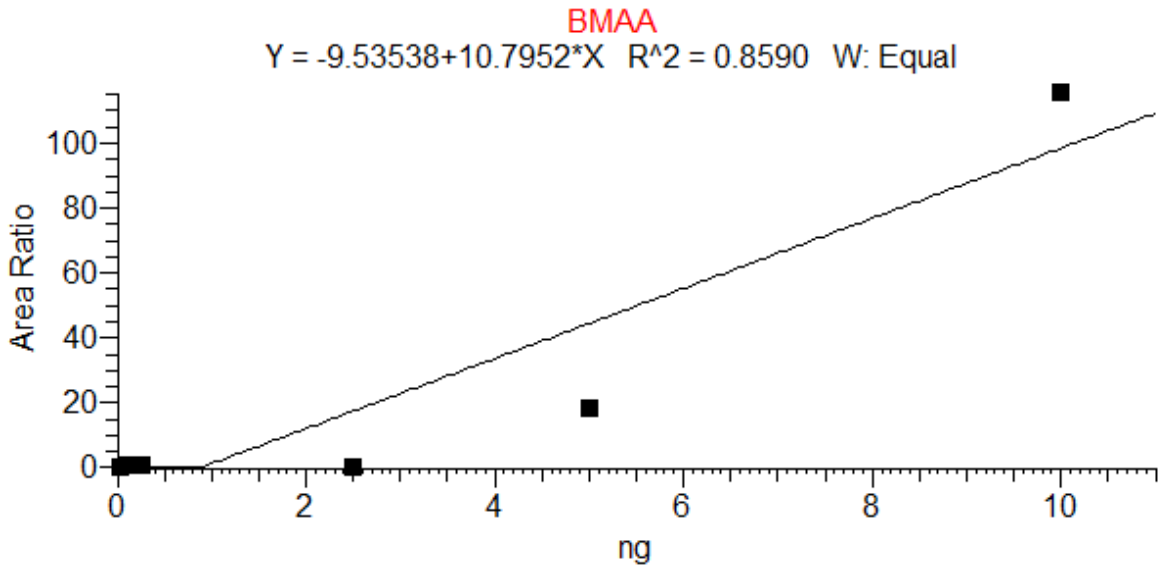


**Figure 1-A.** Calibration curve of BMAA for the nutrient deplete cyanobacteria. The lowest limit of detection on the column was 25-100 pg. Area ratio is the ratio of the external standard (DAB or BMAA) divided by internal standard ($D_3$AB).

## CHAPTER III

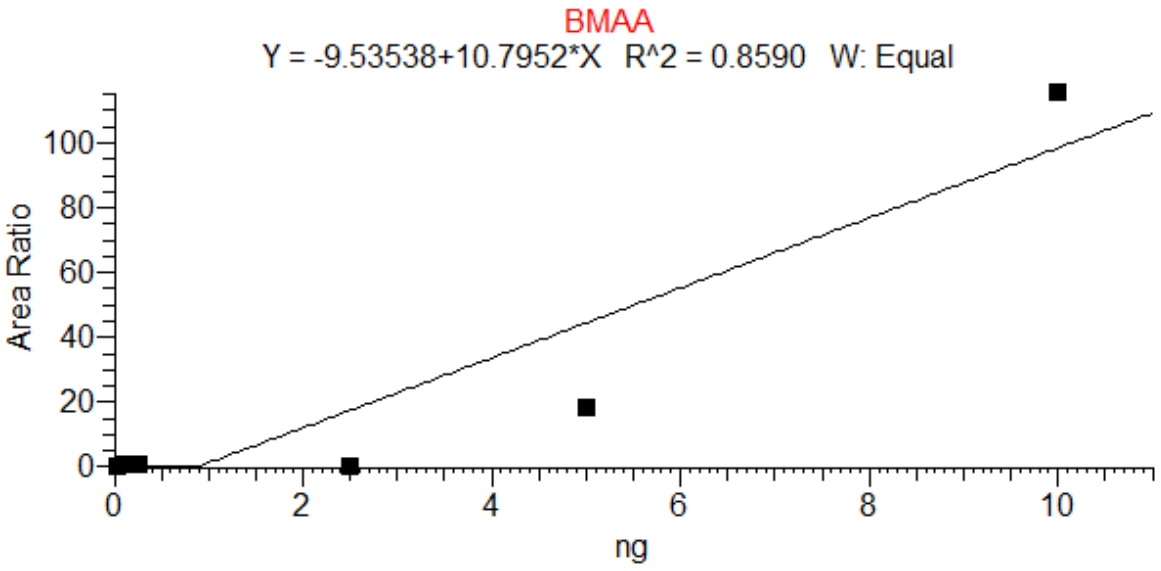


**Figure 2-A.** Calibration curve for the wild cyanobacteria samples and blue crab tissues. The lowest possible limit of detection on the column was 25 pg (0.025 ng). Area ratio is the ratio of the external standard (DAB or BMAA) divided by internal standard ($D_3$AB).

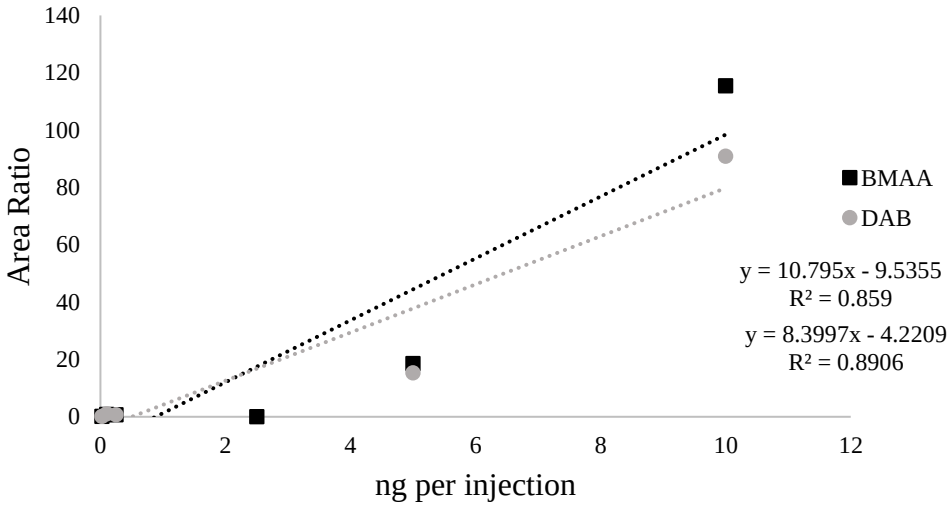


**Figure 3-A.** Calibration curve of BMAA and DAB for the wild cyanobacteria samples and blue crab tissues, showing a limit of detection of 25 pg (0.025ng). Area ratio is the ratio of the external standard (DAB or BMAA) divided by internal standard ($D_3$AB).

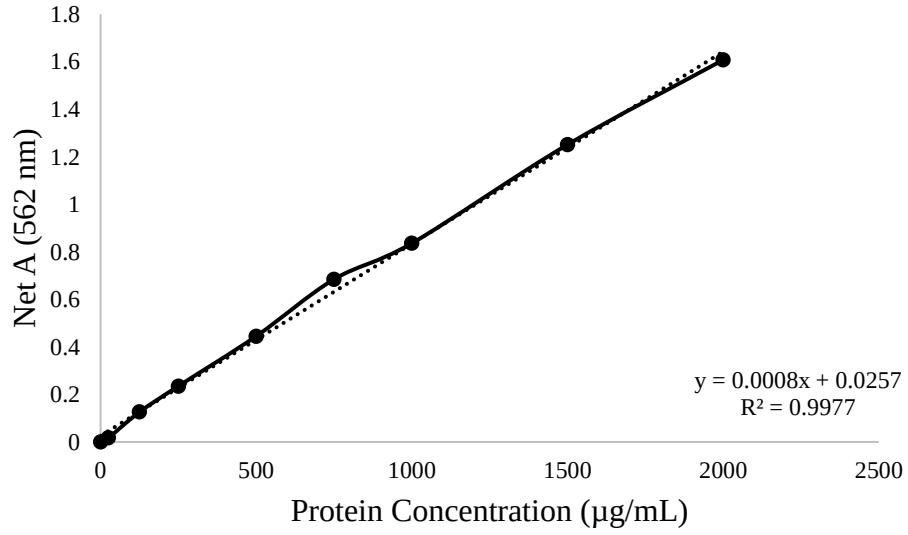


**Figure 4-A.** BSA protein standard curve for protein detection in blue crab tissues. The lowest limit of detection was 25 µg/mL.

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## RESEARCH INTERESTS

2014-2016 Rider University
Advisor Dr. Paul Jivoff: Blue crab biology, physiology, and reproductive system. Analysis of heavy metals in blue crab tissues via ICP-MS
Advisor Dr. Gabriela Smalley: Dinoflagellate growth, culturing, and feeding. Observation under epifluorescent microscopy

2016-2019
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## TEACHING EXPERIENCE

2016-2018 Graduate Teaching Assistant, Old Dominion University (Norfolk, VA)
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## PRESENTATIONS AND PUBLICATIONS

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